Iain J. McEwan · Raj Kumar
Editors

Nuclear Receptors: From Structure to the Clinic

Springer

Nuclear Receptors: From Structure to the Clinic

Iain J. McEwan • Raj Kumar
Editors

Nuclear Receptors: From Structure to the Clinic

Springer

Editors
Iain J. McEwan
School of Medical Sciences
University of Aberdeen
Aberdeen
United Kingdom

Raj Kumar
Department of Basic Sciences
The Commonwealth Medical College
Scranton
Pennsylvania
USA

ISBN 978-3-319-18728-0 ISBN 978-3-319-18729-7 (eBook)
DOI 10.1007/978-3-319-18729-7

Library of Congress Control Number: 2015943689

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Preface

It has been estimated that there are 48 nuclear receptor genes in the human genome. These code for a superfamily of proteins that can regulate gene transcription in response to a wide range of natural and synthetic ligands, including classical steroid hormones, vitamins, intermediate metabolites, xenobiotics and drugs. The first three-dimensional structures for isolated receptor domains appeared 25 years ago with the solution and crystal structures of the glucocorticoid and estrogen receptor DNA binding domains. The intervening years have seen an explosion in structures for the DNA and ligand binding domains of nearly all family members, culminating in the recent emergence of almost complete three-dimensional descriptions for nuclear receptor complexes bound to cognate response elements. These dramatic advances in structural analysis are paralleled by the growing evidence linking nuclear receptor function to normal physiological processes and disease. The insights gained from nuclear receptor structures have the potential to be translated into new drugs for major diseases, including cancer, metabolic syndrome and cardiovascular diseases.

In this book we have brought together a range of review articles to highlight current areas of nuclear receptor research, with the focus on structure and function and translational opportunities for drug discovery. In the first part, the attention is on receptor complexes (Chaps. 6 and 7), allosteric regulation and the role of the intrinsically disordered NTD (Chap. 5) and the role of DNA binding and response element architecture (Chap. 4).

This section also includes reviews on the corticosteroid receptors, glucocorticoid and mineralocorticoid (Chaps. 2 and 3) which are increasingly important clinically in disorders from hypertension and cardiovascular diseases to neurological disorders and cancers. In Part B the focus is on co-regulator protein structure and function. Nuclear receptors act primarily by promoting or disrupting the assembly of productive transcription complexes at target genes. Chapter 9 considers the role of intrinsically disordered structure again, in the assembly of co-repressor complexes by nuclear receptors. In Chap. 8, the attention is on a co-regulator of the androgen receptor that is restricted to primates. In the final section, the emphasis is on the

targeting of nuclear receptors with small molecules that could act in a tissue selective manner (Chap. 11) or target a novel pocket on the surface of the receptor (Chap. 10).

March 2015

Iain J. McEwan, Aberdeen, UK
Raj Kumar, Scranton, USA

Contents

Twenty-five Years of Nuclear Receptor Structure Analysis: From the Laboratory to the Clinic 1
Iain J. McEwan and Raj Kumar

Part I Allosteric Regulation and Nuclear Receptor Complex Dynamics

Corticosteroid Receptors 17
Peter J. Fuller, Jun Yang and Morag J. Young

Glucocorticoid Receptor Structure and Function 41
Raj Kumar and Iain J. McEwan

What Determines the Difference in DNA Binding Between the Androgen and the Glucocorticoid Receptors? 59
Vanessa Dubois, Christine Helsen, Liesbeth Clinckemalie, Lien Spans, Michaël Laurent, Leen Antonio, Thomas Van den Broeck, Stefan Prekovic, Steven Joniau, Dirk Vanderschueren and Frank Claessens

Allosteric Regulation and Intrinsic Disorder in Nuclear Hormone Receptors 73
Jordan T. White, Hesam N. Motlagh, Jing Li, E. Brad Thompson and Vincent J. Hilser

Structural Analyses of Ordered and Disordered Regions in Ecdysteroid Receptor 93
Marek Orłowski, Piotr Dobryszycki, Anna Zoglowek, Joanna Pieprzyk, Katarzyna Bielska, Beata Greb-Markiewicz, Małgorzata Kozłowska, Aneta Tarczewska and Andrzej Ożyhar

Structural Analysis of Heterodimeric Nuclear Receptors: 119
Brice Beinsteiner and Dino Moras

Part II Nuclear Receptor Co-regulatory Protein Interactions

Primate-Specific Multi-Functional Androgen Receptor Coregulator and Proto-Oncogene Melanoma Antigen-A11 (MAGE-A11) 137
Elizabeth M. Wilson

Assembly and Regulation of Nuclear Receptor Corepressor Complexes 155
Christopher J. Millard and John W. R. Schwabe

Part III Taking Nuclear Receptor Structure into the Clinic

Thinking Outside the Box: Alternative Binding Sites in the Ligand Binding Domain of Nuclear Receptors 179
Nerea Gallastegui and Eva Estébanez-Perpiñá

Selective Estrogen Receptor Modulators (SERMs) and Selective Androgen Receptor Modulators (SARMs) 205
Ramesh Narayanan and James T. Dalton

Index 229

Contributors

Leen Antonio Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

Division of Clinical and Experimental Endocrinology, KU Leuven, Leuven, Belgium

Brice Beinsteiner Centre for Integrative Biology (CBI), Department of Integrated Structural Biology, Institute of Genetics and of Molecular and Cellular Biology (IGBMC), Illkirch, France

Institut National de la Santé et de la Recherche Médicale (INSERM) U964, Illkirch, France

Centre National de la Recherche Scientifique (CNRS) UMR 7104, Illkirch, France

Université de Strasbourg, Strasbourg, France

Katarzyna Bielska Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Frank Claessens Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium

Liesbeth Clinckemalie Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

James T. Dalton Dean and Professor of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan, USA

Piotr Dobryszycki Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Vanessa Dubois Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

Eva Estébanez-Perpiñá Department of Biochemistry and Molecular Biology, Institute of Biomedicine of the University of Barcelona (IBUB), University of Barcelona (UB), Barcelona, Spain

Peter J. Fuller MIMR-PHI Institute (Prince Henry's Institute), Clayton, VIC, Australia

Nerea Gallastegui Department of Biochemistry and Molecular Biology, Institute of Biomedicine of the University of Barcelona (IBUB), University of Barcelona (UB), Barcelona, Spain

Beata Greb-Markiewicz Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Christine Helsen Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

Vincent J. Hilser Department of Biology, Johns Hopkins University, Baltimore, MD, USA

T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD, USA

Steven Joniau Division of Urology, KU Leuven, Leuven, Belgium

Małgorzata Kozłowska Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Raj Kumar Department of Basic Sciences, The Commonwealth Medical College, Scranton, PA, USA

Michaël Laurent Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

Division of Gerontology and Geriatrics, KU Leuven, Leuven, Belgium

Jing Li T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD, USA

Iain J. McEwan School of Medical Sciences, Programme of Cell, Developmental and Cancer Biology, University of Aberdeen, Aberdeen, Scotland, UK

Christopher J. Millard Henry Wellcome Laboratories of Structural Biology, Department of Biochemistry, University of Leicester, Leicester, UK

Dino Moras Centre for Integrative Biology (CBI), Department of Integrated Structural Biology, Institute of Genetics and of Molecular and Cellular Biology (IGBMC), Illkirch, France

Institut National de la Santé et de la Recherche Médicale (INSERM) U964, Illkirch, France

Centre National de la Recherche Scientifique (CNRS) UMR 7104, Illkirch, France

Université de Strasbourg, Strasbourg, France

Hesam N. Motlagh T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD, USA

Ramesh Narayanan Preclinical Research and Development, GTx, Inc., Memphis, TN, USA

Marek Orłowski Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Andrzej Ożyhar Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Joanna Pieprzyk Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Stefan Prekovic Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

John W. R. Schwabe Henry Wellcome Laboratories of Structural Biology, Department of Biochemistry, University of Leicester, Leicester, UK

Lien Spans Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

Aneta Tarczewska Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

E. Brad Thompson Center for Nuclear Receptors and Cell Signaling, Department of Biology and Biochemistry, University of Houston, Houston, TX, USA

Prof. Emeritus, University of Texas Medical Branch, Galveston, TX, USA

Thomas Van den Broeck Molecular Endocrinology Laboratory, KU Leuven, Leuven, Belgium

Division of Urology, KU Leuven, Leuven, Belgium

Dirk Vanderschueren Division of Clinical and Experimental Endocrinology, KU Leuven, Leuven, Belgium

Jordan T. White Department of Biology, Johns Hopkins University, Baltimore, MD, USA

Elizabeth M. Wilson Laboratories for Reproductive Biology, Departments of Pediatrics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, USA

Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, USA

Jun Yang MIMR-PHI Institute (Prince Henry's Institute), Clayton, VIC, Australia

Morag J. Young MIMR-PHI Institute (Prince Henry's Institute), Clayton, VIC, Australia

Anna Zoglowek Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

Twenty-five Years of Nuclear Receptor Structure Analysis: From the Laboratory to the Clinic

Iain J. McEwan and Raj Kumar

1 Introduction: Historical Perspective

We see the world in three dimensions and a major achievement of Renaissance painters was to represent the world in two-dimensions through mastering perspective. The ability to convey three-dimensional information has been equally important and revolutionary in biology. It is therefore not surprising that molecular structures, from the iconic DNA double helix to the earliest structural models of myoglobin, have had such an impact, leading to both clearer insights into function and new research paths. The first steroid receptor cDNAs were cloned in the mid-1980s, which led to isolation of cDNAs for related non-steroid receptors and the birth of the nuclear receptor superfamily (Evans 1988). These proteins, generally, act as ligand-dependent transcription factors and known ligands include steroid hormones, vitamins, intermediate metabolites, xenobiotics and drugs. In addition to revealing the common domain organisation of these proteins the availability of receptor cDNAs opened up the possibility for extensive biochemical, biophysical and structural analysis of receptor function. Figure 1 summarizes some of the key structural developments in the last quarter century going from a simple schematic diagram, based on biochemical analysis of the purified rat glucocorticoid receptor (Carlstedt-Duke et al. 1982; Wrange and Gustafsson 1978), to high resolution structures of receptor complexes bound to cognate DNA response elements (Chandra et al. 2008; Lou et al. 2014).

I. J. McEwan (✉)
School of Medical Sciences, Programme of Cell, Developmental and Cancer Biology, University of Aberdeen, Foresterhill, Aberdeen, AB 25 2ZD, Scotland, UK
e-mail: iain.mcewan@abdn.ac.uk

R. Kumar
Department of Basic Sciences, The Commonwealth Medical College, 525 Pine St., Scranton, PA 18509, USA
e-mail: rkumar@tcmc.edu

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_1

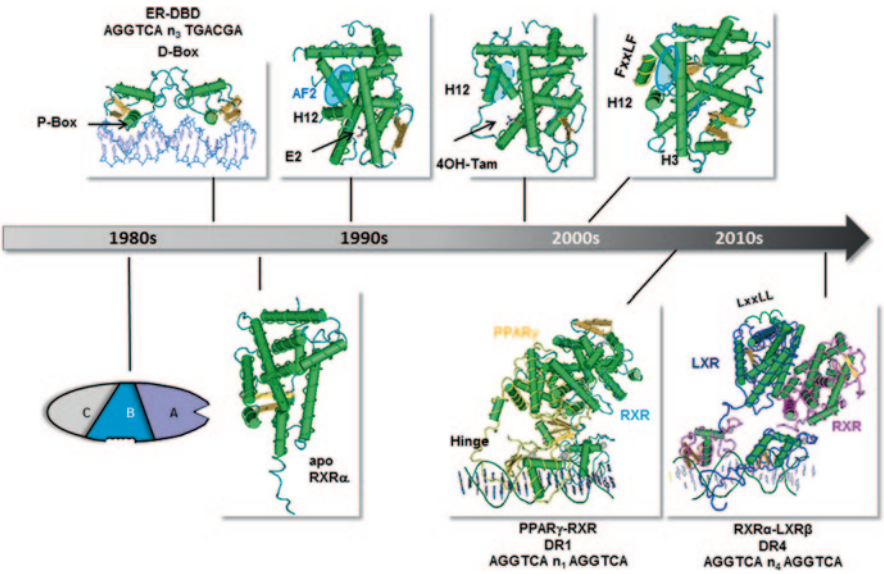


Fig. 1 Timeline of Structural Analysis of members of the Nuclear Receptor Superfamily. In 25 years we have gone from a simple schematic drawing of the domain structure of a steroid receptor (glucocorticoid receptor) derived from biochemical and subsequent receptor cDNA cloning to atomic resolution structures of the isolated DBD and LBD and in the last five years full-length receptor complexes bound to DNA response elements. Most recently the complex also includes a peptide having a LxxLL motif bound to the LBD. ERα DBD, PDB ID: 1HCQ (Schwabe et al. 1993) ; RXRα apo-LBD, PDB 1LBD (Bourguet et al. 1995); ERα LBD, PDB 1ERE; ERα-LBD with tamoxifen bound, PDB 3ERT (Shiau et al. 1998); AR-LDB with co-regulatory peptide, PDB 1XOW (He et al. 2004); PPARγ-RXRα complex, PDB 3E00 (Chandra et al. 2008); and RXRα-LXRβ complex, 4NQA (Lou et al. 2014)

2 Role of Ligands in Regulating Receptor Action

2.1 Hormone/Agonist Binding

The ligand binding domain (LBD) of nuclear receptor is thought to have an open structure in the absence of ligand (Bourguet et al. 1995) and to adopt a more compact structure upon binding agonists, which become buried in the hydrophobic interior (see Brzozowski et al. 1997; He et al. 2004; Renaud et al. 1995; Tanenbaum et al. 1998) (Fig. 1). Nuclear receptors bind a wide range of chemical structures, from steroid hormones to vitamins to xenobiotics, which are accommodated by changes in the volume of the ligand binding pocket, from 0 to over 1500 Å^3, and a small number of receptor-selective amino acid-ligand contacts (reviewed in Gallastegui et al. 2015; Rastinejad et al. 2013).

Structures of the LBD for representatives of different families of nuclear receptors bound to either agonists or antagonists are available; however relative few structures have been reported for the apo-form of nuclear receptors. The canoni-

cal structure for the LBD consists of 11 to 12 α-helices and a variable number of β-strands; for example the estrogen receptor (ER/NR3A1) α-LBD has 11 helices and 2 β-strands (Fig. 1) (reviewed in Rastinejad et al. 2013). The binding and nature of the ligand has a dramatic consequence for the orientation of helix 12, which 'seals' the ligand binding pocket and completes the formation of the AF2 surface when agonists are bound, together with residues in helices 3, 4 and 5 (see Fig. 1) (Gallastegui et al. 2015; Rastinejad et al. 2013). This surface pocket has a hydrophobic interior and typically binds coactivator proteins containing the motif LxxLL (where L is leucine and x is any amino acid). The leucine residues are buried in the pocket and the resulting helix is positioned by a 'charge clamp' on the surface of the LBD involving a lysine residue in helix 4 and a glutamic acid in helix 12 (Darimont et al. 1998; Dubbink et al. 2004; He et al. 2004; Heery et al. 1997; Hur et al. 2004). Genetic studies have helped illustrate a network of intramolecular interactions linking occupancy of the ligand binding pocket with the formation of the AF binding pocket (Nagy and Schwabe 2004). More recently, structural and biophysical studies have highlighted an important allosteric network connecting the AF2 surface with a novel surface pocket termed BF3 (See Sect. 4.3 below). Collectively structural and functional studies of different family members have helped build a picture where binding of agonists cause conformational rearrangements that lead to allosteric regulation of protein-protein interactions and target gene regulation.

2.2 Anatgonist Binding and Action

Antagonists act to competitively inhibiting the binding of natural ligands and so switch off receptor activity. In the presence of an antagonist, for example tamoxifen binding to ERα, the overall fold of the LBD remains similar to that of the estradiol-bound receptor, but there is a dramatic repositioning of helix 12, such that it occludes the AF2 surface and prevents recruitment of co-activator proteins (Fig. 1) (Egea et al. 2000; Huang et al. 2010; Pike et al. 2000).

In addition to simply sterically blocking the AF2 pocket the binding of antagonists may positively lead to the recruitment of co-repressor proteins, through a leucine rich motif, analogous to the LxxLL sequence found in coactivators, LxxxxLxxxI/L (Nagy et al. 1999; Oberoi et al. 2011). Co-repressors proteins were first described for non-steroid members of the family, thyroid hormone receptor (TR) and retinoic acid receptor (RAR), which in the absence of ligand are thought to bind to regulatory elements of target genes and repress transcription.

2.3 DNA Response Elements

The first three-dimensional structures to be published were for the isolated DNA binding domains for the glucocorticoid receptor (GR/NR3C1) and ERα in the absence or presence of DNA response elements (Hard et al. 1990; Schwabe et al.

1990; Schwabe et al. 1993). The core DBD of nuclear receptors consists of fewer than 100 amino acids and folds in to a globular conformation through the binding of two zinc ions; the zinc is coordinated by highly conserved cysteine residues (Fig. 1). Nuclear receptors bind DNA as homodimers (e.g. steroid receptors, ER, GR), heterodimers with retinoid X Receptor (RXR; e.g. the non-steroid ligand binding receptors retinoic acid receptor (RAR), peroxisome proliferator activated receptor (PPAR) and thyroid hormone receptor (TR)) or as monomers (e.g. NGFB-1) (reviewed in Claessens and Gewirth 2004). *In vitro* binding studies identified the six nucleotides 5' AGGTCA 3' or 5' AGAACA 3', arranged as direct and/or inverted repeats with 1 to 5 nucleotides spacers, as nuclear receptor response elements: with the former half-site bound by ER and all non-steroid receptors and the latter by the GR-sub-family of steroid receptors, including androgen (AR/NR3C4), progesterone (PR/NR3C3) and mineralocorticoid (MR/NR3C2) receptors. The first zinc module contains the DNA recognition helix and the 'P-box' residues and makes both direct and water mediated hydrogen bonds between the amino acids and the nucleotide sequence. In addition, there are a number of interactions between amino acid side chains and the phosphate backbone of the DNA. In the case of the ER, and non-steroid nuclear receptors, the P-box residues are Glu, Gly and Ala, while the corresponding amino acids in the GR-subfamily are Gly, Ser and Val (Askew et al. 2007; Green et al. 1988; Green and Chambon 1989). In the second Zn-finger module a five amino acid sequence, the 'D-box', was found to mediate dimerization of the receptor on DNA (Claessens and Gewirth 2004). In the case of steroid receptors this involves direct interactions of the D-box residues of both monomers and for non-steroid receptors forming heterodimers with RXR the D-box of one receptor interacts with amino acids in the first zinc module of the partner (Claessens and Gewirth 2004; Rastinejad et al. 2013).

Although the overall folding of the DBD is very similar for all receptors studied more recent analysis, in particular involving binding to different natural DNA response elements, has highlighted differences that may contribute to receptor-selective function. For example, although limited, so far, to a single structure it appears that the AR-DBD forms a more closely packed dimerization interface than that observed for the GR-DBD. This has been suggested to account, at least in part, for the AR binding and activity on DNA response elements selective for this receptor (Shaffer et al. 2004). Detailed studies from Yamamoto and co-workers (Meijsing et al. 2009; Watson et al. 2013) has directly correlated DNA architecture of natural GR binding sites with structural changes in the receptor DBD and recruitment of co-regulatory protein complexes. A region of the GR-DBD, they have termed the 'leaver arm' (Glu^{450}-Gly-Gln-His-Asn-Tyr^{455}) is found to adopt different conformations depending on the sequence of the response element. The involvement of the 'leaver arm' or corresponding sequences, and the identified allosteric network, in other nuclear receptors is less clear, but a recent study has identified changes in this region in the mineralocorticoid receptor (MR) that may mediate receptor-selective gene regulation (Hudson et al. 2014).

In addition to the sequence comprising the core DBD, the amino acids immediately adjacent, termed the C-terminal extension (CTE), are also known to play

a role in stabilizing DNA binding and/or response element selection. For example in the vitamin D receptor (VDR) and the TR β (NR1A2) the CTE forms an α-helix and is important for heterodimerization with RXR and minor groove DNA contacts (Claessens and Gewirth 2004; Rastinejad et al. 2013; Roemer et al. 2006). In the original structures of the GR and ER and subsequently the AR DBDs the CTE appeared disordered; although mutational studies highlighted a role in DNA binding (Hard et al. 1990; Rastinejad et al. 2013; Schwabe et al. 1990; Schwabe et al. 1993; Shaffer et al. 2004). Interestingly, recent structural analysis of a number of GR-DBD-DNA complexes observed a helical conformation for the CTE (Meijsing et al. 2009). Electron densities were also observed for the first 7 to 8 residues of the PR-CTE, which revealed interactions with both the minor groove and the core PR-DBD (Roemer et al. 2006). Mutational analysis demonstrated that the PR-CTE was necessary for recognizing nucleotides flanking the DNA response element (Roemer et al. 2006). What these different studies illustrate is a growing body of evidence supporting an active role for DNA sequence and conformation in nuclear receptor signalling, beyond simply tethering receptors to target genes. They also raise an intriguing question, namely can this information be exploited transnationally to design more receptor/tissue selective drugs?

3 Allosteric Regulation and Interdomain Communication

As described above, significant insight has been provided by structural analysis of the isolated DNA- and ligand-binding domains. However, such studies are more limited in shedding light on the role of long distance domain interactions and the function of small molecule and DNA ligands in allosteric regulation of receptor activity. Attempts to determine the three-dimensional structure of full-length nuclear receptors have proved challenging due to the size and/or the presence of significant regions of intrinsic disorder.

Recently the first X-ray structures of complexes of PPARγ-RXRα (Chandra et al. 2008), liver X receptor (LXR/NR1H2) β-RXR (Lou et al. 2014) and hepatocyte nuclear factor (HNF)-4α homodimers (Chandra et al. 2013) bound to response elements and with co-regulatory protein peptides have been reported (see Fig. 1). In other studies, the conformation of nuclear receptor complexes have been studied in solution using small angle X-rays (SAX) and fluorescence resonance transfer (FRET) (Rochel et al. 2011), isothermal calorimetry and hydrogen deuterium exchange (HDX) experiments (Chandra et al. 2008; Putcha and Fernandez 2009; Zhang et al. 2011). Collectively these studies have revealed the shape of different receptor complexes and highlighted differences in inter-domain and inter-receptor communication. Figure 2 shows schematic representations of different nuclear receptor complexes bound to direct repeat response elements, having one, three, four or five nucleotide spacer between the half-sites.

A common theme to emerge from these studies is the asymmetric nature of the complexes formed on DNA, with the receptor exhibiting an open or extended con-

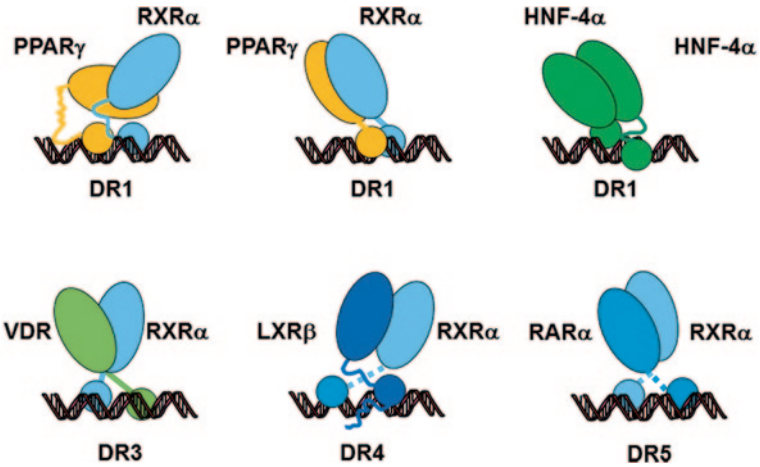


Fig. 2 Schematic representations of nuclear receptor complexes. The LBD and DBD are represented by ovals and circles respectively, while the hinge domain is represented by solid or broken line. See main text for details and references

formation. The exception to this is the crystal structure of PPARγ (NR3C2) and RXRα (NR2B1) bound to a DR1 DNA response element, where the PPAR monomer adopted a 'closed' conformation with extensive interactions between the LBD and the PPAR-DBD and the RXR-LBD, Hinge and DBD: RXR had an 'open' conformation with the hinge region extended, creating a surface for PPAR binding (Chandra et al. 2008) (Figs. 1 and 2). This is in striking contrast to the solution structure for this complex, where both receptors have a more extended, open, conformation (Rochel et al. 2011) (Fig. 2). The different orientations of PPAR and RXR seen in the two studies may just reflect differences in methodologies (crystal v's solution). Alternatively, these observations may illustrate the complexity of conformational space that nuclear receptors occupy and the different conformations that may be adopted, which in turn could underpin gene and/or tissue specific responses.

A further notable feature of the studies described so far is the highly flexible nature of the hinge region of RXRα that allows the binding of different heterodimer partners. In structures involving VDR, LXR and RAR on DR3, 4 and 5 response elements respectively, RXR occupies the 5' half site of the DNA, and there is a similar orientation of the receptor monomers (Fig. 2). In contrast, on a DR1 element RXR adopts the opposite polarity and binds to the 3' half-site (Fig. 2). The complex of LXRβ-RXRα is particularly noteworthy as there was little evidence for inter domain communication, seen in other RXR heterodimer complexes, and a relatively large buried surface representing the heterodimerization surfaces on the LBDs (Lou et al. 2014). The structure also revealed minor groove interactions by a helical CTE and N-terminal loop of the LXRβ monomer, emphasising the importance of amino acid sequences flanking the core DBD in response element recognition and binding.

To-date the only high resolution structure not involving RXR is for a homodimer of HNF-4α bound to a DR1 DNA sequence (Chandra et al. 2013) (Fig. 2). This

structure has been particularly insightful in providing a structural basis for allosteric regulation involving post-translational modifications of the receptor and impact of clinically relevant point mutations. Each monomer adopts a half-site specific orientation, with multiple domain interactions involving the LBDs of both receptors, the DBD of the upstream monomer and the hinge domain of the downstream monomers (Fig. 2): the integration of these interactions is required for high affinity DNA binding and can be modulated by post-translational modifications (Chandra et al. 2013). Methylation of a key arginine residue in the DBD, not directly involved in DNA contacts enhances dimerization of the LBDs and as a result DNA binding. In contrast, phosphorylation of a serine allosteric reduces DNA binding by disrupting domain interactions.

Allosteric regulation of DNA binding, and co-regulatory protein interactions, was also revealed by HDX experiments on the VDR-RXR complex (Zhang et al. 2011). Binding of either ligand, 1,25 vitamin D_3 or 9-*cis*-retinoic acids, alone caused changes in HDX within the cognate receptor LBD and the LBD of the receptor partner. As might be expected, a number of these changes mapped to regions of the receptors involved in heterodimerization. However, there were also perturbations at distant sites, for example 1,25 vitamin D_3 binding caused changes in helix 3 of the RXR partner, suggesting allosteric inter-receptor communication. Most striking was the destabilization of the VDR-DBD by binding of either 1,25-vitamin D_3 or 9-cis-retnoic acid (Zhang et al. 2011). DNA response element recognition and binding also resulted in changes in HDX of both receptors: there was strong protection from solvent exchange for the VDR-DBD/CTE, consistent with more contacts with the DNA. DNA binding, and significantly the architecture of the DNA response element, also led to changes in the LBD of both receptors: the regions affected were the dimerization interface and AF2 surface. A 1:1 complex was formed between a fragment of the coactivator SRC-1 (NCoA1) and VDR-RXR: with VDR binding to the NR3 box and RXR to NR1 box (p160 family of coactivators, NCoA1, 2 and 3, have three LxxLL motifs termed NR box 1 to 3). The nature of the DNA response element was shown to alter the conformation of the AF2 regions such that there was a reduction in VDR and enhancement of RXR interactions with SRC-1 (Zhang et al. 2011). Significantly, a stoichiometry of 1:1 was also observed for the binding of the co-activator Med1 to the in solution structures of RAR-RXR heterodimers bound to a DR5 element (Rochel et al. 2011). Interestingly, in the case of the complex of the constitutive androstane receptor (CAR) and RXR the stoichiometry of SRC-1 binding was governed by the ligand occupancy of the receptors (Pavlin et al. 2014). When both CAR and RXR were bound by ligand two molecules of SRC-1 were present in the complex. In contrast, when only CAR was bound to a ligand, one molecule of SRC-1 was bound.

Taken together the structural analysis of full-length or two-domain receptor proteins, in complex with different DNA response elements, illustrate the complexity of multiple inter/intra-domain interactions and the possible mechanisms of allosteric regulation imparted upon ligand, DNA or co-regulatory protein binding. However, these studies have also emphasised the intrinsic disordered structure of the NTD (reviewed in Kumar and McEwan 2012), as this domain is either missing from

the receptor constructs or fails to yield a stable structure for diffraction studies. The intrinsic disordered nature of the NTD may, at least, partly explain the lack of structural information for steroid receptor complexes, where this domain can represent a significant proportion of the receptor protein.

4 From Bench to Bedside

4.1 Understanding the Genetic Basis for Nuclear Receptor-Dependent Disease

Genetic changes in the nuclear receptor proteins have long been recognised to lead to developmental and metabolic diseases and hormone-dependent cancers (see Table 1). Structural analysis has been useful at explaining the functional consequences of point mutations found in nuclear receptor-dependent diseases. For example, structural modelling and direct crystallography analysis of the H874Y mutation in the AR-LBD, found in prostate cancer, highlighted the basis for altered co-activator protein recruitment to the AF2 surface (Duff and McEwan 2005; He et al. 2006).

Analysis of the wild-type and mutant TRβ LBD structures increased understanding of the basis for thyroid-resistance, identifying mutations that disrupt ligand binding but act in a dominant negative manner. Similarly, mutations in the LBD of PPARγ disrupted the position of helix 12 and resulted in a mutant protein that inhibited the wild-type receptor (Gurnell and Chatterjee 2004; Kallenberger et al. 2003).

More recently, the structure of homodimers of HNF-4α bound to DNA revealed the basis of disease and opened up the possibility for targeted drug discovery. Mutations in HNF-4α have been correlated with MODY and hyperinsulinaemia hypoglycaemia and map to the hinge and LBD or the DBD and LBD respectively (Chandra et al. 2013). In some cases it is clear that the point mutations directly disrupt either DNA or ligand binding. However, examination of the receptor complex has also

Table 1 Examples of inherited diseases resulting from mutations in nuclear receptors

Receptor	Disorder-description
AR	Androgen-insensitivity syndrome: partial or complete disruption of male sex development. Point mutations throughout the receptor coding sequence
GR	Glucocorticoid resistance, associated with fatigue, hypertension, hyperandrogenism and infertility
MR	Psedohypoaldosteronism, disruption of normal salt balance
VDR	Vitamin D-dependent rickets (type II); including hypocalcaemia and alopecia
TRβ	Resistance to thyroid hormone, leading to growth, metabolic and cognitive defects. Mutations localised to the LBD
PPARγ	PPARγ ligand-resistance, associated with insulin resistance and metabolic disease. Mutations in the receptor-LBD
HNF4α	Maturity onset diabetes of the young type I (MODY); early onset type 2 diabetes

shed light on a number of mutations that compromise DNA binding affinity by disrupting the interdomain communication described above. The value here is having a two-domain complex bound to DNA as modelling of the mutations on the isolated domains would not necessarily pick up disruption of the allosteric networks involving interdomain interactions.

4.2 Selective Receptor Modulators

The availability of structures for the LBD has dramatically accelerated progress in the development of selective receptor modulators (SRMs). Nuclear receptor are often expressed in more than one target tissues and while the basis for tissue-selective actions of nuclear receptors are still to be fully understood, it has long been appreciated that targeting receptor in a tissue-specific manner would have enormous health benefits. This can be clearly illustrated with the treatment of hormone-dependent cancers such as breast and prostate. For example, while blunting AR activity is of benefit in the management of diseases such as prostate cancer and benign prostatic hyperplasia, there are also disadvantages as anabolic effects of androgens will also be impaired. Conversely, there are conditions such as hypogonadism and possibly aging, where it is beneficial to give androgens. However, the growth promoting action of androgens on the prostate is of real concern with androgen replacement therapies. To date a number of non-steroidal molecules have been designed and tested as 'androgen selective modulators' (SARMs), in the laboratory and more recently in clinical trials for indicators such as sarcopenia and osteoporosis (reviewed in (Haendler and Cleve 2012; McEwan 2013; Narayanan et al. 2008)

Crystal structures are available for the wild-type AR-LBD bound with the SARMs andarine (Bohl et al. 2008), LGD2226 (Wang et al. 2006) and BMS-564929 (Ostrowski et al. 2007). The canonical agonist bound LBD structure (see Fig. 1) is essentially superimposable for each of these compounds. However, significantly, there are some crucial local conformational changes, notably in the ligand binding pocket, where there is an absence of the hydrogen bonding from T877 in all three structures: this residue makes key interactions with the C17 OH group present in testosterone and DHT (reviewed in McEwan 2013). In the structure with andarine there is also displacement of M745, as a consequence of interactions of the ligand with W741, and I898, which impact on the AF2 pocket (Bohl et al. 2008). Although the mechanism(s) explaining the selective agonist activity of these compounds in tissues such as bone and muscle compared with prostate remains to be determined, it is reasonable to speculate that changes in ligand binding and local conformation are likely to play a role.

A similar picture emerges for targeting of ERα in breast cancer: tamoxifen, an antiestrogen drug approved for use by the FDA in 1977, antagonizes estrogen signalling in the breast but acts as an agonist in the endometrium. Binding of the active antiestrogen, 4hydroxy-tamoxifen, causes displacement of helix 12 in the LBD, resulting in steric hindrance of the AF2 pocket and inhibition of estrogen signalling (Fig. 1) (McDonnell 2000; Pike et al. 1999). In contrast the NTD AF1 domain is

not inhibited by tamoxifen, which is thought to explain the agonist activity of this drug. There is therefore a clear clinic need for selective ER modulators (SERMs), that retain beneficial actions of estrogen in bone, cardiovascular tissues but lack proliferative activity in breast and uterus. The situation with estrogen signalling is further complicated by the existence of different isoforms (i.e. ERα and ERβ), which exhibit tissue selective expression and function (Nettles et al. 2004).

4.3 Thinking Outside the Ligand Binding Pocket

Traditionally, drug discovery for nuclear receptors has focused naturally enough on the ligand binding pocket and the design of small molecules that could compete or replace endogenous ligands. Although highly successful it is also realised that resistance to these drugs can impair there efficacy and dose and systemic actions can lead to serious side-effects, again limiting their usefulness in the clinic. For this reason, there is increasing interest in targeting other regions of the receptor protein. One of the best described sites has been termed 'BF3' and was originally identified in structural studies of the AR-LBD, and found to be a site for small-molecule binding. In the presence of DHT, small molecules were observed to occupy BF3, which consists of residues from helices 1, 3 (+ loop) and 9 (Estebanez-Perpina et al. 2007) . Furthermore, the binding of small molecules such as triiodothyroacetic acid (TRIAC), thyroid hormone and flufenamic acid modulated the binding of peptides to the AF2 surface, providing evidence for local structural and allosteric changes in the absence of gross conformational changes in the LBD. Interestingly, in addition to blocking coactivator binding to AF2 these compounds were found to increase turnover of the receptor protein (Estebanez-Perpina et al. 2007) . Recent work has expanded the analysis to include other steroid receptors and virtual screening and functional assays has led to the identification of additional classes of chemicals binding to the AR-BF3 surface (Lack et al. 2011).

Collectively these studies, originating from structural studies, have highlighted the potential to identify molecules that can inhibit directly or allosterically the AF2 surface of the AR and thereby modulate receptor activity.

5 Conclusions and Future Perspectives

The last 25 years have seen tremendous progress in our understanding of both the structure and function of nuclear receptors. From high resolution structures derived from X-ray crystallography and NMR spectroscopy to the more recent application of SAXS, HDX and cryo-electron microscopy, structural analysis provides information on receptor complex shape and domain communication.

In this book we have endeavoured to bring together a range of review articles to high lightcurrent areas of nuclear receptor research, with the focus on structure

and function and translational opportunities for drug discovery. In the first part, the attention is on receptor complexes (Chaps. 6 and 7), allosteric regulation and the role of the intrinsically disordered NTD (Chap. 5) and the role of DNA binding and response element architecture (Chap. 4).

This section also includes reviews on the corticosteroid receptors, GR and MR (Chaps. 2 and 3) which are increasingly important clinically in disorders from hypertension and cardiovascular diseases to neurological disorders and cancer. In Part 2 the focus is on co-regulator protein structure and function. Nuclear receptor act primarily by promoting or disrupting the assembly of productive transcription complexes at target genes. Chapter 9 considers the role of intrinsically disordered structure again, in the assembly of co-repressor complexes by nuclear receptors. In Chap. 10, the attention is on a co-regulator of the AR that is restricted to primates. In the final section the emphasis is on the targeting of nuclear receptors with small molecules that could act in a tissue selective manner (Chap. 11) or target a novel pocket on the surface of the receptor (Chap. 10).

So what can we expect in the future? It seems likely that there will be further structures determined for full-length or at least two-domain receptor complexes, bound to DNA and co-regulatory proteins. A clear gap in our current understanding is the lack of a structure for a full-length steroid receptor member of the family. Given the size and the relatively large intrinsically disordered NTD this poses a significant challenge. However, an intermediate step could be the structure of a two-domain (DBD-hinge-LBD) steroid receptor homo-dimer bound to DNA. As discussed above such a structure could provide invaluable information on inter and intra-domain interactions and identify allosteric networks between the LBD/hormone and DBD/DNA. A further challenge is to understand the conformational space occupied by the NTD of nuclear receptors and how this is regulated by co-regulatory protein binding and post-translational modifications (see Kumar and McEwan 2012 and references therein). It is therefore significant that the first cryo-EM structure for the DNA bound ERα/SRC-3/CBP complex has just been published (Yi et al. 2015).The structure is relatively low resolution, 25 Å, and so lacks detailed information. However, together with supporting biochemical data a number of interesting conclusions can be drawn. The DNA-bound ERα recruits two molecules of SRC-3, which in turn make multiple interactions with CBP: of particular note are the potential conformational changes between the isolated CBP and CBP bound in the complex and acetylation of histone 3. Another significant feature of the complex is the location of the ERα-AF1 domain, using monoclonal antibodies, within the complex and the potential for inter-domain communication (Yi et al. 2015).

A third challenge is to then translate the information on nuclear receptor structures into improved therapies for some of the major health concerns such as metabolic syndrome, cardiovascular disease and hormone-dependent cancers.

Acknowledgments Work in IJM laboratory is supported by funding from the Chief Scientists Office (CSO) of the Scottish Government.

References

Askew EB, Gampe RT Jr, Stanley TB, Faggart JL, Wilson EM (2007) Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. J Biol Chem 282(35): 25801–25816

Bohl CE, Wu Z, Chen J, Mohler ML, Yang J, Hwang DJ, Mustafa S, Miller DD, Bell CE, Dalton JT (2008) Effect of B-ring substitution pattern on binding mode of propionamide selective androgen receptor modulators. Bioorg Med Chem Lett 18:5567–5570

Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. Nature 375:377–382

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758

Carlstedt-Duke J, Okret S, Wrange O, Gustafsson JA (1982) Immunochemical analysis of the glucocorticoid receptor: identification of a third domain separate from the steroid-binding and DNA-binding domains. Proc Natl Acad Sci USA 79:4260–4264

Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, Burris TP, Rastinejad F (2008) Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. Nature 456:350–356

Chandra V, Huang P, Potluri N, Wu D, Kim Y, Rastinejad F (2013) Multidomain integration in the structure of the HNF-4alpha nuclear receptor complex. Nature 495:394–398

Claessens F, Gewirth DT (2004) DNA recognition by nuclear receptors. Essays Biochem 40:59–72

Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ, Yamamoto KR (1998) Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 12:3343–3356

Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J (2004) Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132–2150

Duff J, McEwan IJ (2005) Mutation of Histidine 874 in the Androgen Receptor Ligand-Binding Domain Leads to Promiscuous Ligand Activation and Altered p160 Coactivator Interactions. Mol Endocrinol 19:2943–2954

Egea PF, Klaholz BP, Moras D (2000) Ligand-protein interactions in nuclear receptors of hormones. FEBS Lett 476:62–67

Estebanez-Perpina E, Arnold LA, Nguyen P, Rodrigues ED, Mar E, Bateman R, Pallai P, Shokat KM, Baxter JD, Guy RK, Webb P, Fletterick RJ (2007) A surface on the androgen receptor that allosterically regulates coactivator binding. Proc Natl Acad Sci USA 104:16074–16079

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889–895

Gallastegui N, Mackinnon JA, Fletterick RJ, Estebanez-Perpina E (2015) Advances in our structural understanding of orphan nuclear receptors. Trends Biochem Sci 40:25–35

Green S, Chambon P (1989) Chimeric receptors used to probe the DNA-binding domain of the estrogen and glucocorticoid receptors. Cancer Res 49:2282s–2285s

Green S, Kumar V, Theulaz I, Wahli W, Chambon P (1988) The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity. EMBO J 7:3037–3044

Gurnell M, Chatterjee VK (2004) Nuclear receptors in disease: thyroid receptor beta, peroxisome-proliferator-activated receptor gamma and orphan receptors. Essays Biochem 40:169–189

Haendler B, Cleve A (2012) Recent developments in antiandrogens and selective androgen receptor modulators. Mol Cell Endocrinol 352:79–91

Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson JA, Kaptein R (1990) Solution structure of the glucocorticoid receptor DNA-binding domain. Science 249:157–160

He B, Gampe RT, Jr, Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM (2004) Structural basis for androgen receptor interdomain and coactivator in-

teractions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425–438
He B, Gampe RT, Jr, Hnat AT, Faggart JL, Minges JT, French FS, Wilson EM (2006) Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity. J Biol Chem 281:6648–6663
Heery DM, Kalkhoven E, Hoare S, Parker MG (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736
Huang P, Chandra V, Rastinejad F (2010) Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol 72:247–272
Hudson WH, Youn C, Ortlund EA (2014) Crystal structure of the mineralocorticoid receptor DNA binding domain in complex with DNA. PLoS ONE 9:e107000
Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ (2004) Recognition and accommodation at the androgen receptor coactivator binding interface. PLoS Biol 2:E274
Kallenberger BC, Love JD, Chatterjee VK, Schwabe JW (2003) A dynamic mechanism of nuclear receptor activation and its perturbation in a human disease. Nat Struct Biol 10:136–140
Kumar R, McEwan IJ (2012) Allosteric modulators of steroid hormone receptors: structural dynamics and gene regulation. Endocr Rev 33:271–299
Lack NA, Axerio-Cilies P, Tavassoli P, Han FQ, Chan KH, Feau C, LeBlanc E, Guns ET, Guy RK, Rennie PS, Cherkasov A (2011) Targeting the binding function 3 (BF3) site of the human androgen receptor through virtual screening. J Med Chem 54:8563–8573
Lou X, Toresson G, Benod C, Suh JH, Philips KJ, Webb P, Gustafsson JA (2014) Structure of the retinoid X receptor alpha-liver X receptor beta (RXRalpha-LXRbeta) heterodimer on DNA. Nat Struct Mol Biol 21:277–281
McDonnell DP (2000) Selective estrogen receptor modulators (SERMs): a first step in the development of perfect hormone replacement therapy regimen. J Soc Gynecol Investig 7:S10–S15
McEwan IJ (2013) Androgen receptor modulators: a marriage of chemistry and biology. Future Med Chem 5:1109–1120
Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324:407–410
Nagy L, Schwabe JW (2004) Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci 29:317–324
Nagy L, Kao HY, Love JD, Li C, Banayo E, Gooch JT, Krishna V, Chatterjee K, Evans RM, Schwabe JW (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev 13:3209–3216
Narayanan R, Mohler ML, Bohl CE, Miller DD, Dalton JT (2008) Selective androgen receptor modulators in preclinical and clinical development. Nucl Recept Signal 6:e010
Nettles KW, Sun J, Radek JT, Sheng S, Rodriguez AL, Katzenellenbogen JA, Katzenellenbogen BS, Greene GL (2004) Allosteric control of ligand selectivity between estrogen receptors alpha and beta: implications for other nuclear receptors. Mol Cell 13:317–327
Oberoi J, Fairall L, Watson PJ, Yang JC, Czimmerer Z, Kampmann T, Goult BT, Greenwood JA, Gooch JT, Kallenberger BC, Nagy L, Neuhaus D, Schwabe JW (2011) Structural basis for the assembly of the SMRT/NCoR core transcriptional repression machinery. Nat Struct Mol Biol 18:177–184
Ostrowski J, Kuhns JE, Lupisella JA, Manfredi MC, Beehler BC, Krystek SR, Jr, Bi Y, Sun C, Seethala R, Golla R, Sleph PG, Fura A, An Y, Kish KF, Sack JS, Mookhtiar KA, Grover GJ, Hamann LG (2007) Pharmacological and x-ray structural characterization of a novel selective androgen receptor modulator: potent hyperanabolic stimulation of skeletal muscle with hypostimulation of prostate in rats. Endocrinology 148:4–12
Pavlin MR, Brunzelle JS, Fernandez EJ (2014) Agonist ligands mediate the transcriptional response of nuclear receptor heterodimers through distinct stoichiometric assemblies with coactivators. J Biol Chem 289:24771–24778
Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618

Pike AC, Brzozowski AM, Hubbard RE (2000) A structural biologist's view of the oestrogen receptor. J Steroid Biochem Mol Biol 74:261–268

Putcha BD, Fernandez EJ (2009) Direct interdomain interactions can mediate allosterism in the thyroid receptor. J Biol Chem 284:22517–22524

Rastinejad F, Huang P, Chandra V, Khorasanizadeh S (2013) Understanding nuclear receptor form and function using structural biology. J Mol Endocrinol 51:T1–T21

Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. Nature 378:681–689

Rochel N, Ciesielski F, Godet J, Moman E, Roessle M, Peluso-Iltis C, Moulin M, Haertlein M, Callow P, Mely Y, Svergun DI, Moras D (2011) Common architecture of nuclear receptor heterodimers on DNA direct repeat elements with different spacings. Nat Struct Mol Biol 18:564–570

Roemer SC, Donham DC, Sherman L, Pon VH, Edwards DP, Churchill ME (2006) Structure of the progesterone receptor-deoxyribonucleic acid complex: novel interactions required for binding to half-site response elements. Mol Endocrinol 20:3042–3052

Schwabe JW, Neuhaus D, Rhodes D (1990) Solution structure of the DNA-binding domain of the oestrogen receptor. Nature 348:458–461

Schwabe JW, Chapman L, Finch JT, Rhodes D (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75:567–578

Shaffer PL, Jivan A, Dollins DE, Claessens F, Gewirth DT (2004) Structural basis of androgen receptor binding to selective androgen response elements. Proc Natl Acad Sci USA 101:4758–4763

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937

Tanenbaum DM, Wang Y, Williams SP, Sigler PB (1998) Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. Proc Natl Acad Sci USA 95:5998–6003

Wang F, Liu XQ, Li H, Liang KN, Miner JN, Hong M, Kallel EA, van Oeveren A, Zhi L, Jiang T (2006) Structure of the ligand-binding domain (LBD) of human androgen receptor in complex with a selective modulator LGD2226. Acta Crystallogr Sect F Struct Biol Cryst Commun 62:1067–1071

Watson LC, Kuchenbecker KM, Schiller BJ, Gross JD, Pufall MA, Yamamoto KR (2013) The glucocorticoid receptor dimer interface allosterically transmits sequence-specific DNA signals. Nat Struct Mol Biol 20:876–883

Wrange O, Gustafsson JA (1978) Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. J Biol Chem 253:856–865

Yi P, Wang Z, Feng Q, Pintilie GD, Foulds CE, Lanz RB, Ludtke SJ, Schmid MF, Chiu W, O'Malley BW (2015) Structure of a biologically active estrogen receptor-coactivator complex on DNA. Mol Cell 57(6):1047–1058

Zhang J, Chalmers MJ, Stayrook KR, Burris LL, Wang Y, Busby SA, Pascal BD, Garcia-Ordonez RD, Bruning JB, Istrate MA, Kojetin DJ, Dodge JA, Burris TP, Griffin PR (2011) DNA binding alters coactivator interaction surfaces of the intact VDR-RXR complex. Nat Struct Mol Biol 18:556–563

Part I
Allosteric Regulation and Nuclear Receptor Complex Dynamics

Corticosteroid Receptors

Peter J. Fuller, Jun Yang and Morag J. Young

1 Introduction

The two corticosteroid receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) are important well validated therapeutic targets. The role of the GR in mediating the response of synthetic glucocorticoids in the treatment of a range of inflammatory, autoimmune or malignant conditions has been extensively canvassed (Busillo and Cidlowski 2013). Increasingly the importance of antagonism at the MR in the treatment of an expanding range of diseases, particularly cardiovascular disease, has also been appreciated (Pitt et al. 1999, 2003; Zannad et al. 2010). The MR is generally recognised as the "aldosterone receptor", the mediator of the regulation of epithelial sodium transport by the adrenal steroid hormone aldosterone. It was, however, originally identified as the type 1, corticosteroid receptor (Feldman et al. 1973). This reflects the sometimes overlooked fact that both receptors bind cortisol (corticosterone in rodents) but only the MR binds aldosterone (Rogerson et al. 1999). The MR is thus unique amongst the steroid receptors in having two physiological ligands (Fig. 1). The distinction "mineralocorticoid" versus "type 1 corticosteroid" is conferred at a tissue level by the enzyme 11β hydroxysteroid dehydrogenase type 2 (11βHSD2) which in epithelial tissues, the vasculature and discrete subpopulations of hypothalamic neurones (Geerling and Loewy 2009) confers aldosterone specificity (Odermatt and Kratschmar 2012). The MR is however expressed in a diverse range of tissues including macrophages and cardiomyocytes (Rickard et al. 2009, 2012) where it plays a fundamental role in cellular function and pathology; in these tissues it is undoubtedly acting as a receptor for cortisol. In some non-epithelial tissues, such as neurones and cardiomyocytes, there is evidence that physiological glucocortocoids can antagonise aldosterone (Gomez-Sanchez et al. 1990; Sato and Funder 1996; Mihailidou 2006).

P. J. Fuller (✉) · J. Yang · M. J. Young
MIMR-PHI Institute (Prince Henry's Institute), 27-31 Wright Street,
Clayton, VIC 3168, Australia
e-mail: peter.fuller@princehenrys.org

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_2

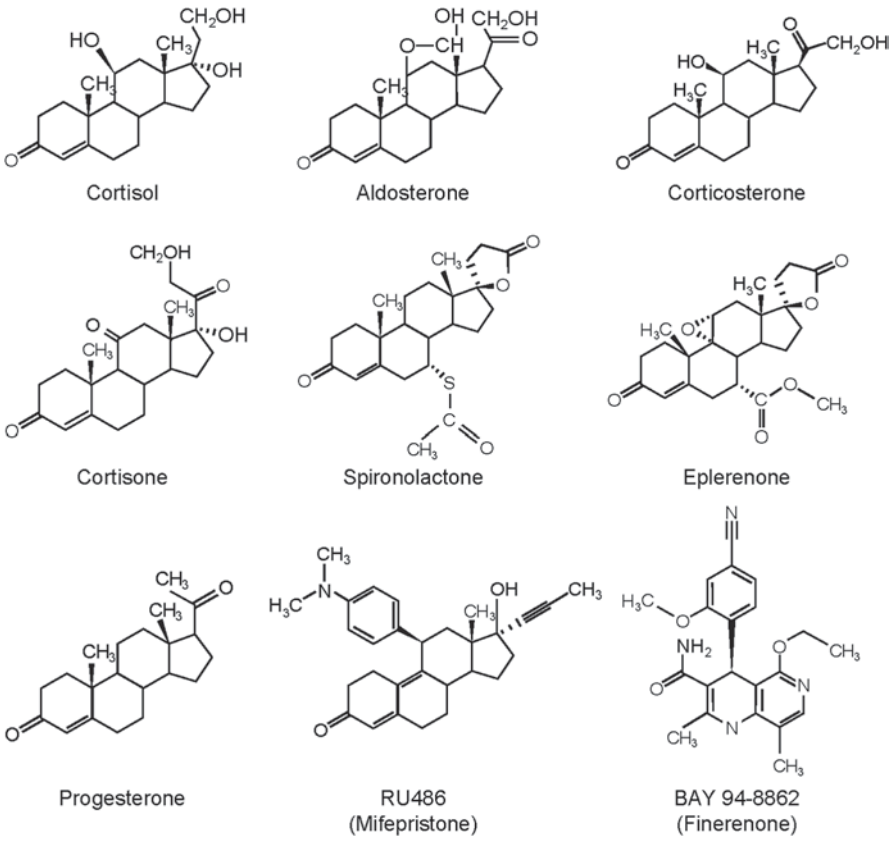


Fig. 1 Chemical structures of the corticosteroid receptor agonist and antagonist ligands discussed

This chapter will explore the structural determinants of function within the corticosteroid receptors, primarily focusing on the MR but drawing analogy with the GR as appropriate, noting also that the GR is extensively discussed in Chap. 3 of this volume. Critical to understanding structure-function relationships in the MR are the interactions made both internally and with other factors (Yang and Fuller 2012), many of which will influence the conformation of the MR or depend on a specific conformation.

2 Mineralocorticoid Receptor Structure

The human MR (NR3C2) is the longest of the nuclear receptors containing 984 amino acids, by contrast the human GR (NR3C1) contains 777 amino acids. As with other members of the NR family, the MR has three major functional domains: an N-terminal domain (NTD), a central DNA-binding domain (DBD) and a C-terminal

ligand-binding domain (LBD) (Arriza et al. 1987). The MR-NTD (602 amino acids) is the longest of the steroid hormone receptors and it shares little sequence homology with these other receptors (Lavery and McEwan 2005). The MR-NTD contains three regions to which an activation function (AF) has been ascribed: AF1a (amino acids 1-169), MD (middle domain, amino acids 247–385) and AF1b (amino acids 451–602) (Fischer et al. 2010; Fuse et al. 2000; Govindan and Warriar 1998; Pascual-Le Tallec et al. 2003). A central inhibitory region (amino acids 163–437) has also been identified (Pascual-Le Tallec et al. 2003) although it may reflect the ability of the MD to recruit corepressors (Fischer et al. 2010). Vlassi et al. (2013) report that the inhibitory domain (amino acids 168–445 in the human MR) contains 15 tandem repeats of ~10 amino acids, an ensemble that is highly conserved in evolution and is not observed in the GR. The authors predict that these repeats will form a β-solenoid surface which may play a role in dimerisation or intermolecular hydrophobic interactions of MR. Aside from regulating transactivation, the NTD also interacts with the LBD as discussed below (Rogerson and Fuller 2003). The NTD is considered to be intrinsically disordered in the absence of binding partners allowing structural flexibility for diverse protein interactions (McEwan et al. 2007). AF-1b has recently been shown to adopt a stable secondary structure and can interact with protein targets in the absence of induced folding (Fischer et al. 2010), highlighting the complexity of this region.

The DBD of 66 amino acids is highly conserved across the nuclear receptor superfamily. It contains two zinc ions tetrahedrally coordinating four cysteine residues and residues important for DNA recognition and binding, as well as for receptor homo- and hetero-dimerisation (Pippal and Fuller 2008) . It is linked to the LBD by a hinge region of 61 amino acids that may play a role in receptor dimerisation (Savory et al. 2001).

The LBD has a canonical structure that is conserved across the NR superfamily (Tsuji 2013). The MR LBD of 251 amino acids is organised in eleven αhelices (labelled by convention 1–12; helix 2 is unstructured in the SHRs) and four β-strands forming three anti-parallel layers (Bledsoe et al. 2005; Fagart et al. 2005; Huyet et al. 2007; Li et al. 2005). It contains a ligand-dependent activation function 2 (AF-2) made up of helices 3, 4, 5 and 12. The LBD interacts with chaperone proteins in the absence of ligand and undergoes conformational change upon ligand binding to form AF-2, a hydrophobic cleft on the surface of the LBD which binds coregulators.

3 Structural Determinants of Ligand-Specificity

Within the steroid receptor sub-family of the nuclear receptor superfamily (GR, MR, AR and PR), the amino acid sequence of the ligand-binding domains share 50–60 % identity. This sequence conservation has a functional correlate in that the MR antagonist spironolactone is also an antagonist at the AR and agonist at the PR, progesterone is an antagonist at the MR and GR, while RU486 is a PR and GR antagonist, yet all is not promiscuity. To understand the structural basis of how the MR is able to bind aldosterone and cortisol, yet the GR binds only cortisol, Roger-

son et al. (1999) took a chimeric approach. Such approaches had previously been used to explore the structural basis of differences in ligand-binding affinity for the same receptor across species (Benhamou et al. 1992; Keightley et al. 1998). Sixteen MR:GR chimeric LBD were created with the three break points being at regions of high identity, the study having been initiated prior to publication of the crystal structures of the GR (Bledsoe et al. 2005) and MR (Bledsoe et al. 2002; Fagart et al. 2005; Li et al. 2005). Despite this, the structural integrity was preserved in that all chimeras were able to bind cortisol albeit with a spectrum of affinities. The full-length receptor containing the LBD chimeras was expressed in CV-1 cells with an MMTV-reporter in a conventional transactivation assay. The N-terminus and DBD were derived from the GR, a strategy based largely on the fact that the GR N-terminus is more active in a transactivation assay than the N-terminus of the MR (the reverse is true of the respective LBD) (Rupprecht et al. 1993; Lim-Tio et al. 1997). Of the 16 chimeras, those that contained MR sequences in the second segment (amino acids 804–870) were both transcriptionally active and bound aldosterone (Rogerson et al. 1999). Curiously this was not true for cortisol where transactivation only occurred when the second and fourth regions were derived from the same receptor, i.e. both MR or both GR; this clearly argues for a difference in the LBD interactions between aldosterone and cortisol. The region of MR 804–874 also conferred binding specificity on spironolactone (Rogerson et al. 2003) and eplenerone (Rogerson et al. 2004) neither of which bind with significant affinity to the GR. Rogerson et al. (2007) subsequently used the same approach with smaller regions of MR 804–874 to identify a 25 amino acid region (MR 820–844) containing four critical residues that together confer the ability to bind or not bind aldosterone on the MR and GR respectively (Fig. 2).

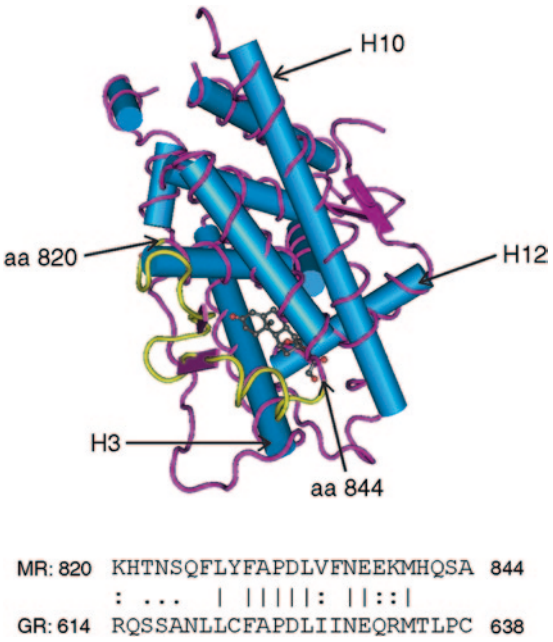


Fig. 2 MR LBD crystal structure with the region MR820-844 highlighted. α-helices are represented by the rods and β-sheets as ribbons (based on Bledsoe et al. 2005—Protein Data Bank 2AA2). Helices 3, 10 and 12 are labelled as such. Below is the amino sequence for the 820–844 region of the MR and the equivalent region of the GR which is primarily a loop between helicies 5 and 6

Other studies using AR:PR chimeras (Vivat et al. 1997), GR:PR chimeras (Robin-Jagerschmidt et al. 2000) and MR:GR chimeras (Martinez et al. 2005) identified regions responsible for ligand specificity that correspond to the MR 820–844 region. Amino acids 820–844 cover parts of helix 5, helix 6 and the loop between. Only one amino acid, the phenyalanine at position 829 in the MR has been described as contributing to the ligand-binding pocket of the MR LBD (Bledsoe et al. 2005); however this amino acid is also found at the equivalent position in the GR. Amino acids 820–844 are otherwise predicted to lie on the surface of the LBD, particularly the H5-H6 loop. This finding is reminiscent of our findings for the guinea-pig GR, which relative to the human GR, is resistant to cortisol due to a five amino acid difference in the region between H1 and H3, which is another unstructured loop predicted to be on the surface of the GR LBD (Bledsoe et al. 2002). These observations are consistent with the notion that it is an external interaction of these regions that mediates ligand sensitivity and selectivity.

4 Chaperone Proteins

In the absence of ligand, MR (and GR) are complexed with cytoplasmic chaperone proteins (Rafestin-Oblin et al. 1989), including the 90 kDa (hsp90), which is crucial for high-affinity ligand-binding by the MR (Binart et al. 1995; Huyet et al. 2012). The MR is also known to interact with the 70 kDa (hsp70), small acidic protein p23 and tetratricopeptide repeat (TPR, 34 amino acids repeated in tandems)-domain proteins such as FK506-binding proteins (FKBPs), cylophilins (CyPs) or serine/threonine protein phosphatase 5 (PP5) (Bruner et al. 1997; Pratt and Toft 1997; Rafestin-Oblin et al. 1989). This complex maintains the MR in an appropriate conformation for high-affinity ligand- binding and prevents its nonspecific activation (Faresse et al. 2010). The other co-chaperones play a dynamic role in determining ligand affinity (Huyet et al. 2012). Binding of hsp90 to the MR is mediated, at least in part, by the C-terminal of the hinge region (Huyet et al. 2012). The absence or antagonism of hsp90 leads to the polyubiquitylation and proteasomal degradation of MR via the ubiquitin-protein ligase, COOH-terminus of hsp70-interacting protein (CHIP) (Faresse et al. 2010).

Dissociation of hsp90 and other chaperone proteins from the MR is thought to take place in the cytoplasm upon aldosterone binding prior to the nuclear translocation of MR. However evidence has shown that hsp90 is actually required for the efficient nuclear transport of the MR by linking it to the FKBP52-dynein motor protein complex (Galigniana et al. 2010a, b). This large heterocomplex most likely passes intact through the nuclear pore since the MR can be recovered bound to hsp90 immediately after nuclear translocation while MR transformation appears to occur within the nucleus 10–15 min after steroid binding. Grossman et al. (2012) have recently demonstrated that MR homodimerisation occurs in the nucleus when agonist is bound rather than in the cytoplasm. FKBP51, which does not bind dynein and negatively regulates MR action, dissociates from the MR upon aldosterone binding so as to permit the recruitment of FKBP52 (Galigniana et al. 2010b;

Gallo et al. 2007). This differential regulation of dynein interaction by FKBP51 and FKBP52 is also seen with the GR (Wochnik et al. 2005). The cochaperone SGTA (small glutamine-rich tetratricopeptide repeat-containing protein alpha) represses receptor activity for AR, PR and GR through an interaction with FKBP52, yet is without effect on the MR (Paul et al. 2014). In the case of the GR, it has been well established that the relative concentrations of FKBP51 and FKBP52 will determine the affinity of the unliganded receptor for steroid with FKBP52 promoting a higher binding affinity. It is these interactions for instance that appear critical to the reduced binding affinity seen for the guinea pig GR (Cluning et al. 2013). This argues that a helix 1-helix 3 loop interaction with FKBP51/52 plays a role in defining the conformation and hence the ligand-binding affinity of the unliganded GR (Cluning et al. 2013).

These observations reinforce a critical limitation in our understanding of the structure of steroid receptors given the lack of a crystal structure for the unliganded receptor. Whilst there is good evidence from other nuclear receptors for a critical shift in helix 12 with a "tighter" conformation of the helices when ligand is bound, the current crystal structure provides only a snapshot of the "final resting place". The critical interaction between steroid and unliganded receptor is not captured, which is an inherent limitation to any attempt at rationale drug design.

5 Structural Determinants of Agonism Versus Antagonism

The classical MR antagonist spironolactone is seen as a "passive antagonist" where competition for binding occurs but the conformation of the LBD is largely unaltered, and the internal conformation changes needed for transactivation fail to occur (Bledsoe et al. 2005). Spironolactone is in fact a weak agonist/predominant antagonist; the agonist response can be enhanced by cyclic AMP analogues, in a cell and promoter dependent context and by MR coactivators (Nordeen et al. 1995; Massaad et al. 1997; Rogerson et al. 2014). Eplerenone, the other MR antagonist currently in clinical practice, is a derivative of spironolactone and as such exhibits a similar mechanism of action. Although eplerenone has a lower affinity for the MR than spironolactone, it is much more selective, being almost devoid of activity at the other steroid receptors (Fagart et al. 2010).

Insights into the structural basis of the antagonism have been drawn from the observations that a single serine to leucine substitution at position 810, which was identified in a kindred with hypertension (Geller et al. 2000; Zhang et al. 2005), made spironolactone and progesterone agonists as it does the normally inactive metabolite of cortisol, cortisone (Rafestin-Oblin et al. 2003). Zhang and Geller (2008) argue that the helix 3-helix 5 interaction is critical both in the MR and across steroid receptors. These helices contain residues which form hydrogen bonds with the steroid A and D rings (the latter also interacts with residues in helix 12) to bind the steroid with high affinity. This positioning of the steroid in the binding pocket

with the critical points of contact being at both ends of the steroid, holding the steroid rather like a "chicken in a rotisserie" as first noted with the estrogen receptor α (ERα) (Brzozowski et al. 1997). Zhang and Geller (2008) have argued that the Ser 810 Leu mutation results in an enhanced ligand-independent interaction of helix 3 and helix 5 promoting transactivation by otherwise antagonist ligands, a postulate that is at least partially supported by subsequent solution of the crystal structure of the MR LBD containing these and other mutations (Fagart et al. 2005; Bledsoe et al. 2005; Li et al. 2005). Curiously spironolactone can also be rendered agonist by changes in other amino acids. For both the trout and zebra-fish MR, spironolactone (and progesterone) are agonist, yet they do not contain a substitution at Ser810 (Sturm et al. 2005; Pippal et al. 2011) . Whilst this helix 3-helix 5 interaction is clearly of considerable importance, these studies and indeed other structural analyses are confounded by several limitations: (1) there is a tendency to focus on a specific region, e.g. the helix 5–6 loops (Rogerson et al. 1999; 2007), the helix 3–5 interactions (Geller et al. 2000; Zhang et al. 2005; Zhang and Geller 2008) etc., yet all are potentially relevant and perhaps can't be viewed in isolation; (2) binding is somehow taken as being synonymous with transactivation yet these can clearly be dissociated (Rogerson et al. 1999), indeed antagonism may be seen as a dissociation of binding and transactivation while absence of binding renders any other considerations null and void; (3) although the data derived from the published MR LBD crystal structures are of enormous value (Bledsoe et al. 2005;, Li et al. 2005; Fagart et al. 2005), they all contain various LBD mutations to facilitate formation of stable crystals (Zhang and Geller 2008), which results in an agonist conformation irrespective of the ligand; (4) specificity and sensitivity in the context of binding are arguably the same thing, i.e. specificity purely reflects differences in affinity; and (5) in the context of agonism versus antagonism the studies tend to overlook (Zhang and Geller 2008) the importance of the interactions formed with helix 12 as highlighted by Auzou et al. (2000) in which correct positioning of helix 12 is absolutely essential for the AF-2 function and its consequent interaction with coactivator molecules.

6 Interdomain Interactions

Despite a view which arose from the early studies following the cloning of the steroid receptors (Green and Chambon 1987) that the primary domains are largely modular and can be "mixed and matched", it is now clear that interdomain interactions may be critical to function. A ligand-dependent interaction between the N-terminal domain and the C-terminus/LBD has been extensively characterised for the AR (Langley et al. 1995; He et al. 1999; Zhou et al. 1995). This interaction has been shown to be fundamental to AR function, indeed mutations causing androgen insensitivity syndrome in humans have been identified that selectively abrogate the N/C-interactions (Thompson et al. 2001; Quigley et al. 2004) . An N/C-interaction has also been described for the PR (Tetel et al. 1999) and ERα (Mètivier et al. 2002).

Some years ago we identified an equivalent ligand-dependent interaction for the MR (Rogerson and Fuller 2003). Curiously this interaction is not seen for the GR. This aldosterone-induced interaction is antagonised by spironolactone and eplerenone and rather surprisingly by cortisol and deoxycorticosterone, clear evidence that these MR agonists induce a different conformation in MR LBD to that induced by aldosterone. The interaction is conserved across evolution in that it is also observed with the zebra fish MR (Pippal et al. 2011). Studies using fluorescence response energy transfer (FRET) have shown for the AR that the interaction can be intramolecular when the AR is a monomer and intermolecular when it is a dimer (Klokk et al. 2007; Schaufele et al. 2005). In the AR, FxxLF-like motifs in the N-terminus bind to the AF-2 region of the AR LBD competing for binding of the classical LxxLL-motif found in steroid receptor co-activators. The structural determinants appear to be different for the MR in that although the interaction is a direct protein-protein interaction between the N- and C-terminal domains (Pippal et al. 2009) , the MR N-terminal domain lacks FxxLF or LxxLL motifs and indeed deletion studies suggest more than one region is involved (Pippal et al. 2009). Four sumoylation sites identified in the human MR (3 of which are conserved in the zebra fish MR) do not compromise the N/C-interaction when inactivated by mutating the lysines to arginine. The unstructured nature of the N-terminal domain, where composition rather than sequence may be more important, makes the dissection of the interactions particularly challenging. On the C-terminal side, inactivation of AF-2 function attenuates but does not eliminate the N/C-interaction (Rogerson and Fuller 2003), this is in contrast to the interaction of the MR LBD with LxxLL motif containing coactivators where the interaction is completely eliminated (Rogerson et al. 2014).

7 DNA Binding

The primary mode of action of MR is as a transcription factor with its DBD binding to a hormone response element (HRE) in the promoter of target genes to mediate transcription (Lombes et al. 1993). Specific HREs have been characterized for ER, PR, GR and AR, but a selective MR response element has yet to be described. That the MR interacts with diverse GR response elements is not surprising in that it shares 94 % identity with the GR DBD (Arriza et al. 1987; Funder 1993; Lombes et al. 1993). These HREs typically consist of two receptor binding half-sites with the consensus seuences 5'-TGTTCT-3' arranged as an inverted palindrome separated by three nucleotides (Luisi et al. 1991) although two novel HREs upstream of the endothelin-1 gene have been described for the MR and GR that are half-sites separated by eight nucleotides (Stow et al. 2009). The MR has also been shown to interact directly with the promoter of the epidermal growth factor receptor (EGFR) via a region that does not contain the canonical GRE (Grossmann et al. 2007).

The sequence of the specific HRE also plays a role in determining the response. The GRE has been shown to regulate GR conformation to determine selective co-factor interactions (Meijsing et al. 2009). This also has been reported for the ER (Heery et al. 1997; Loven et al. 2001) and AR (Brodie and McEwan 2005) with their respective HRE. The response element bound by the AR has been shown to induce conformational changes in the NTD (Brodie and McEwan 2005; Geserick et al. 2003). The HRE is therefore an important interacting partner with unique regulatory functions. Whether the same applies to the MR remains to be determined.

In addition to binding as homodimers to HRE, the MR is also capable of forming heterodimers with other steroid receptors, in particular the GR, to offer additional transcriptional control. Trapp et al. (1994) demonstrated a direct MR-GR interaction with a dissociation rate of the heterodimer that was slower than either of the homodimers resulting in a synergistic effect on transcription activation. By contrast Liu et al. (1995) reported that MR/GR heterodimers inhibited transcription. Inhibition of the neuronal serotonin receptor in response to corticosterone was greater for MR/GR heterodimers than for MR or GR homodimers alone (Ou et al. 2001). The GR-LBD has been shown to be required for heterodimerisation with the MR (Savory et al. 2001). Coordinated signaling by GR and MR may be important in tissues such as the hippocampus where there is an abundance of both receptors, and a recent study highlighted the potential role of MR and GR heterodimerisation in the kidney (Ackermann et al. 2010). Co-localisation of the MR and GR was observed in all cell types of the aldosterone-sensitive distal nephron and they underwent distinct patterns of subcellular localisation in response to corticosteroids (Ackermann et al. 2010). MR-GR heterodimers have been detected by FRET in hippocampal cells where the level of heterodimerisation is regulated by fluctuations in cortisol concentrations in response to circadian rhythm and stress (Nishi 2010).

Repression of gene expression is well characterised for other steroid hormone receptors, either by competition with other transcription factors for overlapping DNA binding sites or by direct protein-protein interaction in a DNA-independent manner (Fig. 3) (Cato et al. 1992). The GR for example mediates transcriptional repression through a direct interaction with activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) (Jonat et al. 1990; Ray and Prefontaine 1994). The C-terminal zinc finger of the MR-DBD, which is identical to the GR, is reported to also inhibit NFκB by a direct interaction with its RelA subunit (Liden et al. 1997). Reciprocal inhibition of MR-mediated transcription by NFκB has been observed (Kolla and Litwack 2000), however the physiological relevance of these observations remains to be determined. In other systems the MR appears to enhance NFκB and AP-1 activity (Fiebeler et al. 2001). Conversely, the MR is unable to repress AP-1 induced transactivation (Cato et al. 1992; Pearce and Yamamoto 1993). These differences in MR and GR transactivation at the same response element lead Pearce (1994) to speculate that “transcription of genes linked to putative composite MREs is either activated or repressed depending on the composition of nonreceptor factors bound at the elements”.

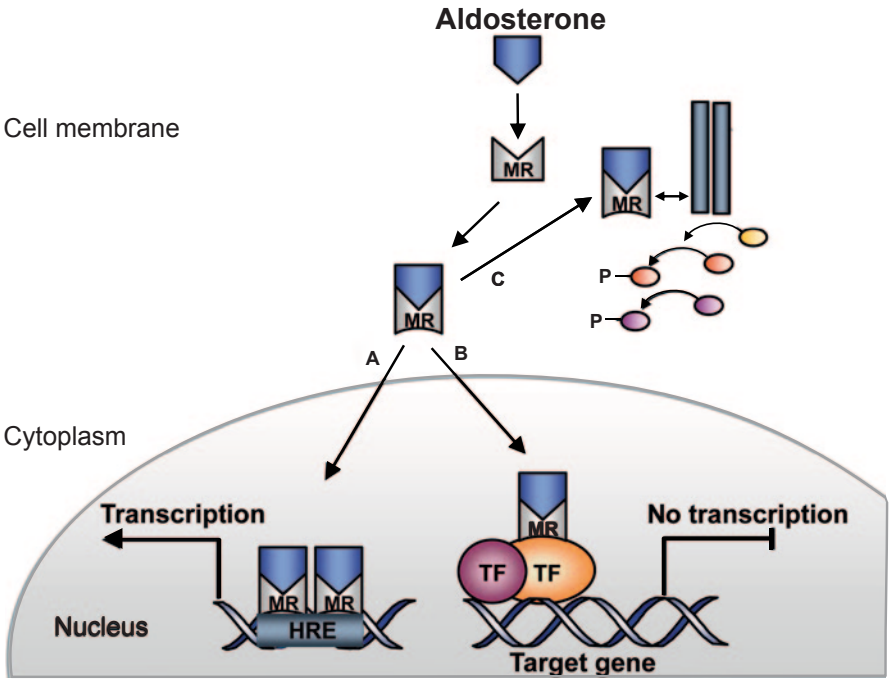


Fig. 3 Schematic of MR signalling. Aldosterone binding to the cytoplasmic MR leads to a conformational change and nuclear transfer where the MR regulates gene expression through binding to a specific response element (HRE) and interactions with coregulating molecules (**a**). An alternative putative mechanism of MR-mediated gene regulation (**b**) involves interaction with other transcription factors (TF). Rapid signalling involves an interaction of the MR with other signalling pathways at the cell surface to trigger second messenger mediated signalling pathways (**c**)

8 Co-Activators and Co-Repressors

Once bound to the regulatory region of a target gene, the liganded-receptor complex is interacting with both the chromatin and the transcriptional apparatus through co-regulatory molecules which are often part of a larger transcriptional complex (McInerney et al. 1998). These complexes perform many of the functions needed for gene expression, including chromatin remodelling, histone modification, initiation of transcriptional elongation of RNA transcripts and termination of transcription (Auboeuf et al. 2007; O'Malley 2007). Over 400 coregulators have been described for the nuclear receptor superfamily (Bulynko and O'Malley 2011) although to date less than 20 have been described for the MR (Yang and Young 2009; Yang et al. 2012). The well characterised coactivators steroid coactivator-1 (SRC-1), SRC-2, SRC-3 and the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) have all been shown to interact with the AF-2 region of the MR LBD via classical LxxLL motifs as they do for the other receptors. In no case do they show ligand-discriminant transactivation. Indeed two studies which explore inter-

actions between known coactivator-derived LxxLL motifs and the MR LBD using either a mammalian-2 hybrid assay (Hultman et al. 2005) or an alpha-screen (Li et al. 2005) found a relatively small number of interactions, all of which interacted in the presence of both cortisol and aldosterone. In contrast, using a yeast-2-hybrid screen with the MR LBD, we identified a novel LxxLL motif-dependent MR coactivator, tesmin, that shows ligand-discriminant transactivation for aldosterone versus cortisol in that under identical conditions, the cortisol-mediated transactivation by the MR is not enhanced by the presence of tesmin yet it is for aldosterone (Rogerson et al. 2014). We have also identified MR interacting proteins using phage-display with and without LxxLL motifs which are ligand-discriminant (Yang et al. 2011; Yang et al. 2014). Although these studies again highlight the difference in the conformation of the MR LBD induced by cortisol and aldosterone, the structural determinants of these differences remain to be determined.

Other groups have sought MR-specific coactivators by screening with the N-terminal region. A yeast two-hybrid screen of a human kidney cDNA library using the MR-NTD as bait identified the elongation factor ELL (eleven-nineteen lysine-rich leukemia) as an MR-specific interacting partner (Pascual-Le Tallec et al. 2005). ELL interacts exclusively with the AF-1b region of the MR and is selective in potentiating MR-mediated transactivation while repressing GR transactivation. ELL is co-expressed with the MR in the cortical collecting duct cells of the human kidney and is rapidly upregulated by aldosterone (Pascual-Le Tallec et al. 2005). This may be relevant in tissues with coexpression of both receptors (Bookout et al. 2006). Ubiquitin-like protein SUMO-1 (small ubiquitin-related modifier-1) conjugating enzyme (Ubc9), is involved in the sumoylation process whereby Ubc9 interacts with the MR-NTD and potentiates aldosterone-dependent MR transactivation independent of its sumoylation activity (Yokota et al. 2007). SRC-1 is recruited simultaneously and can synergistically potentiate MR-mediated transcription with Ubc9, suggesting that Ubc9 mediates its effect by binding SRC-1 and recruiting other coactivators. This is supported by the co-localisation of MR, Ubc9 and SRC-1 in mouse kidney collecting duct cell nuclei (Yokota et al. 2007).

Protein inhibitor of activated signal transducer and activator of transcription 1 (PIAS1), another sumoylation enzyme, has also been found to interact with the MR-NTD (Pascual-Le Tallec et al. 2003). PIAS1 significantly inhibits aldosterone-dependent MR-mediated transactivation. The repressive effect of PIAS1 is partly mediated by sumoylation of the MR. PIAS1 contains three LxxLL motifs, all of which have been shown to enhance transactivation mediated by the AR and GR (Tan et al. 2000). PIAS3, like PIAS1, also interacts with the MR-NTD and represses MR-induced transactivation in a neural cell line (Tirard et al. 2004). Differential modulation of MR- and GR-mediated transcription by PIAS1 and PIAS3 offers another mechanism by which the two receptors can achieve receptor-specific effects.

The earliest identified corepressors, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), interact with the MR-LBD via their CoRNR boxes and have been shown to attenuate aldosterone-dependent MR-mediated transactivation by inducing histone deacetylase activity (Wang et al. 2004). These corepressors were identified through their role in

transcriptional repression of nuclear receptors or when bound to synthetic antagonists in the absence of ligand (Chen and Evans 1995; Horlein et al. 1995). However, recent data has shown that NCoR is recruited by the MR upon aldosterone binding but not upon binding of spironolactone, a steroidal MR antagonist, or BR-4628, a newly synthesised non-steroidal MR antagonist (Fagart et al. 2010).

NF-YC is a subunit of the heterotrimeric transcription factor NF-Y which recognises a CCAAT box motif found in the RNA polymerase II initiation site in many eukaryotic promoters and activates transcription (Nakshatri et al. 1996). It interacts with the MR-NTD and represses MR transactivation in a dose- and agonist-concentration dependent manner (Murai-Takeda, et al. 2010). The mechanism of repression may involve disruption of the N/C-interaction. NF-YC has no effect on GR, AR or PR-mediated transactivation.

9 Post-Translational Modification

Post-translational modifications such as receptor phosphorylation, sumoylation and ubiquitination (Fig. 4) have been described as significant modulators of NR signaling by altering their protein-protein interactions, DNA binding and degradation, although information relating to the MR is sparse (Viengchareun et al. 2007).

Phosphorylation is ubiquitous among all eukaryotic cellular proteins and critically regulates protein structure, localisation and stability (Lalevee et al. 2010). The MR contains tyrosine, serine and threonine residues in its NTD and LBD which represent potential phosphorylation sites (Hirschberg et al. 2004; Viengchareun et al. 2007). In this context, the MR 820–844 region has been brought into focus by a recent study which reports that phosphorylation of serine 843 in the human MR inactivates the MR (Shibata et al. 2013) They argue for a physiological role for serine 843 phosphorylation where in the intercalated cells of the distal nephron but not the principal cells, hyperkalaemia increases phosphorylation through an unspecified ki-

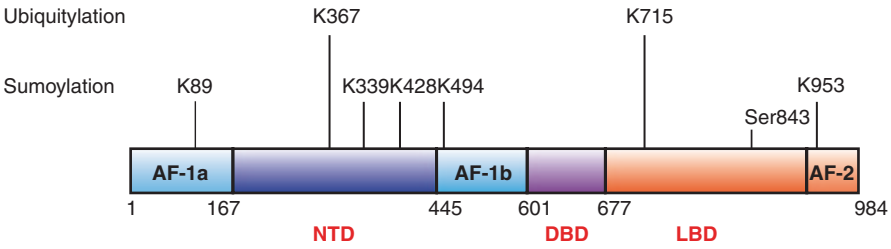


Fig. 4 Post-translational modifications of the MR. The human MR sequence is shown with domains (N-terminal domain—NTD; DNA-binding domain-: DBD; ligand binding domain—LBD) indicated, as are the positions of the described activating functions (AF). The lysine (K) residues subject sumoylation and ubiquitylation are indicated as is serine 843 which is inactivated by phosphorylation (Shibata et al. 2013). A more extensive list of phosphorylation sites of uncertain biological significance can be found in Shibata et al. (2013)

nase. Conversely angiotensin II promotes sodium and hydrogen ion flux by increasing dephosphorylation of serine 843. The authors propose that this allows a degree of dissociation of the two feedback loops mediated by aldosterone through the MR: (i) volume homeostasis through the renin-angiotensin system which increases aldosterone synthesis and hence sodium retention; and (ii) potassium homeostasis where hyperkalaemia promotes aldosterone synthesis with consequent renal potassium secretion. Whilst sodium retention and potassium secretion are coupled in the principal cells, this is not the case in the intercalated cells.

Mutations in the serine/threonine-rich sequences of the MR nuclear localisation signal suggest a role for phosphorylation in receptor subcellular shuttling (Walther et al. 2005). An interaction between the MR and protein kinase D1 (PKD1) modulates the subcellular trafficking of the MR and stabilises its nuclear localisation (McEneaney et al. 2010). Cyclin-dependent kinase 5 (CDK5) has also been shown to phosphorylate three serine and threonine residues within the NTD of the MR in an aldosterone-dependent fashion to suppress MR transcriptional activity (Kino et al. 2010).

Sumoylation involves the covalent attachment of small ubiquitin-related modifiers (SUMO) to the lysine residues of substrate proteins. It is catalysed by a cascade of enzymes including the E1-activating enzyme, E2-conjugating enzyme Ubc9 and E3-ligase (Seeler and Dejean 2003). This process occurs at the consensus motif ψKxD/E (ψ is a hydrophobic residue, K is the lysine targeted by SUMO-1, x is any amino acid and D/E is an acidic residue). These residues were originally identified as the "synergy control motif" in the GR-NTD which acted as a negative regulatory element of GR transactivation (Iniguez-Lluhi and Pearce 2000). Via the same motif, sumoylation of the AR, PR and GR has repressive effects on their transactivation (Abdel-Hafiz et al. 2002; Poukka et al. 2000b; Tian et al. 2002). The MR contains the most number of sumoylation sites, with four in the NTD and one in the LBD (Poukka et al. 2000a) which interacts with components of the sumoylation machinery, including Ubc9 (SUMO-1 conjugating enzyme) and SUMO-1 (Tirard et al. 2007). Ubc9 has previously been identified as a coactivator of the MR, independent of its sumoylation ability (Yokota et al. 2007). In contrast, SUMO-1 binding mediates repression of transcriptional synergy at the MR while sumoylation deficient MR displayed reduced nuclear mobility and therefore longer periods of promoter occupancy and transcriptional activation (Tirard et al. 2007). The exact biological significance of sumoylation is unclear but the highly conserved nature of these motifs and their presence in the negative regulatory regions of many transcription factors suggest they play an important functional role in controlling receptor activity (Viengchareun et al. 2007).

Ubiquitylation and proteasomal degradation have been reported for the MR (Tirard et al. 2007; Yokota et al. 2004) but again the functional significance is unknown. The GR undergoes proteasomal degradation and clearance from the nucleus at the trough of each corticosterone pulse within hippocampal cells but the MR remains intact in the nucleus (Conway-Campbell et al. 2007). This may be a further method of differential regulation of the GR and MR in tissues where they are coexpressed.

10 Structural Determinants of Rapid Signaling

An extensive literature exists with respect to so-called non-genomic or rapid signaling by aldosterone (Dooley et al. 2012; Grossman et al. 2012; Grossman and Gekle 2012 Groeneweg et al. 2012). In most cases this involves the classical MR acting at the cell membrane or in the cytoplasm to trigger a signaling cascade (Karst et al. 2005; Baudrand et al. 2014) Descriptions of distinct cell membrane receptors for aldosterone unrelated to the MR require confirmation although several recent studies have focused on the G-protein estrogen receptor (GPER), a G-protein coupled receptor (Feldman and Gros 2013). The relative significance of these signaling mechanisms vis-à-vis the classical genomic pathway remains to be determined.

11 Structure and the Clinic

The significance of the GR and its therapeutic modulation has been covered in Chap. 3. The MR is similarly important as a therapeutic target. Whilst epithelial MR activity is vital for the maintenance of extracellular fluid volume and potassium homeostasis, its activation in the cardiovascular system (Young and Rickard 2012; McCurley and Jaffe 2012) renal parenchyma (Blasi et al. 2003) macrophages (Rickard et al. 2009) and adipocytes (Guo et al. 2008; Marzolla et al. 2012) are often detrimental to health.

The efficacy of traditional MR antagonists such as spironolactone and eplerenone is best documented in the cardiovascular system with abundant clinical evidence for their benefits in the management of heart failure (Pitt et al. 1999; Pitt et al. 2003; Zannad et al. 2010). MR antagonists have also been shown to reduce vascular injury, inflammation and albuminuria in patients with diabetes mellitus and nephropathy (Shavit et al. 2012) and decrease obesity-related insulin resistance in obese diabetic mice (Hirata et al. 2009).

The main factor limiting the wider use of MR antagonists to attain these clinical benefits is their lack of tissue specificity and consequent electrolyte disturbances due to epithelial MR blockade (Juurlink et al. 2004). In recent years, several classes of non-steroidal MR antagonists, such as the pyrazoles (Meyers et al. 2010), benzoxazin-3-one derivatives (Hasui et al. 2011) and dihydropyridine calcium channel blockers (Dietz et al. 2008); Fagart et al. 2010) have been discovered which are highly potent and selective for the MR. BR-4628, a dihydropyridine-derived compound is highly selective for the MR with minimal effects on the GR, PR, AR and calcium channels. It blocks not only the wild-type MR but also the gain-of-function S810L mutant MR that is paradoxically activated by spironolactone (Geller et al. 2000; Fagart et al. 2010). Spironolactone acts as a “bulky” passive antagonist that interferes with AF-2 domain formation by de-establishing helix 12 thereby preventing it from recruiting coregulators. However, these new agents appear not to have solved the problem of renal epithelial MR antagonism and the adverse effect of hyperkalemia (Kolkhof and Borden 2012). Another novel compound, BAY 94-8862,

was developed using a medicinal chemistry optimisation program and has been found to offer cardiac protection with a reported lower incidence of hyperkalemia (Pitt et al. 2013). The exact mechanism of action of BAY 94-8862 has not been described.

An understanding of the MR structure-function relationships that underlie the tissue-specific actions of MR will be crucial to the development of a newer generation of tissue-selective MR antagonists. Furthermore, the distinction between aldosterone- and cortisol-induced MR conformations may be manipulated to achieve even more targeted modulation of MR activity.

12 Conclusions

The corticosteroid receptors have complex structures that remain to be fully elucidated. In particular, the MR contains a long N-terminal domain that is intrinsically disordered and contributes to a myriad of protein interactions. More studies are required to characterise the structure and function of the MR in the presence of various interacting partners, such as different DNA response elements, chaperone proteins and coregulators so as to unravel the complex network of interactions that regulate MR-mediated gene expression and allow a more targeted approach to the development of selective MR modulators.

Acknowledgements The authors wish to thank Claudette Thiedeman for preparation of the manuscript. This work was supported by the National Health & Medical Research Council of Australia through a Senior Principal Research Fellowship to PJF (#1002559). MIMR-PHI Institute is supported by the Victorian Government's Operational Infrastructure Program.

References

Abdel-Hafiz H, Takimoto GS, Tung L, Horwitz KB (2002) The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. J Biol Chem 277:33950–33956

Ackermann D, Gresko N, Carrel M, Loffing-Cueni D, Habermehl D, Gomez-Sanchez C, Rossier BC, Loffing J (2010) In vivo nuclear translocation of mineralocorticoid and glucocorticoid receptors in rat kidney: differential effect of corticosteroids along the distal tubule. Am J Physiol Renal Physiol 299:F1473–F1485

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268

Auboeuf D, Batsche E, Dutertre M, Muchardt C, O'Malley BW (2007) Coregulators: transducing signal from transcription to alternative splicing. Trends Endocrinol Metab 18:122–129

Auzou G, Fagart J, Souque A, Hellal-Lévy C, Wurtz JM, Moras D, Rafestin-Oblin ME (2000) A single amino acid mutation of ala-773 in the mineralocorticoid receptor confers agonist properties to 11beta-substituted spirolactones. Mol Pharmacol 58(4):684–691

Baudrand R, Pojoga LH, Romero JR, Williams GH (2014) Aldosterone's mechanism of action: roles of lysine-specific demethylase 1, caveolin and striatin. Curr Opin Nephrol Hypertens 37:23–32

Benhamou B, Garcia T, Lerouge T, Vergezac A, Gofflo D, Bigogne C, Chambon P, Gronemeyer H (1992) A single amino acid that determines the sensitivity of progesterone receptors to RU486. Science 255:206–209

Binart N, Lombes M, Baulieu EE (1995) Distinct functions of the 90 kDa heatshock protein (hsp90) in oestrogen and mineralocorticosteroid receptor activity: effects of hsp90 deletion mutants. Biochem J 311(Pt. 3):797–804

Blasi ER, Rocha R, Rudolph AE, Blomme EA, Polly ML, McMahon EG (2003) Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats. Kidney Int 63(5):1791–1800

Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE (2002) Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell 110:93–105

Bledsoe RK, Madauss KP, Holt JA, Apolito CJ, Lambert MH, Pearce KH, Stanley TB, Stewart EL, Trump RP, Willson TM, Williams SP (2005) A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. J Biol Chem 280:31283–31293

Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 126:789–799

Brodie J, McEwan IJ (2005) Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. J Mol Endocrinol 34:603–615

Bruner KL, Derfoul A, Robertson NM, Guerriero G, Fernandes-Alnemri T, Alnemri ES, Litwack G (1997) The unliganded mineralocorticoid receptor is associated with heat shock proteins 70 and 90 and the immunophilin FKBP-52. Recept Signal Transduction 7:85–98

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389(6652):753–758

Bulynko YA, O'Malley BW (2011) Nuclear receptor coactivators: structural and functional biochemistry. BioChemistry 50:313–328

Busillo JM, Cidlowski JA (2013) The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. Trends Endocrinol Metab 24(3):109–119

Cluning C, Ward BK, Rea SL, Arulpragasam A, Fuller PJ, Ratajczak T (2013) The helix 1–3 loop in the glucocorticoid receptor LBD is a regulatory element for FKBP cochaperones. Mol Endocrinol 27(7):1020–1035

Cato ACB, König H, Ponta H, Herrlich P (1992) Steroids and growth promoting factors in the regulation of expression of genes and gene networks. J Steroid Biochem Mol Biol 43:63–68

Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457

Conway-Campbell BL, McKenna MA, Wiles CC, Atkinson HC, de Kloet ER, Lightman SL (2007) Proteasome-dependent down-regulation of activated nuclear hippocampal glucocorticoid receptors determines dynamic responses to corticosterone. Endocrinology 148:5470–5477

Dietz JD, Du S, Bolten CW, Payne MA, Xia C, Blinn JR, Funder JW, Hu X (2008) A number of marketed dihydropyridine calcium channel blockers have mineralocorticoid receptor antagonist activity. Hypertension 51:742–748

Dooley R, Harvey BJ, Thomas W (2012) Non-genomic actions of aldosterone: from receptors and signals to membrane targets. Mol Cell Endocrinol 350:223–234

Fagart J, Huyet J, Pinon GM, Rochel M, Mayer C, Rafestin-Oblin M-E (2005) Crystal structure of a mutant mineralocorticoid receptor responsible for hypertension. Nat Struct Mol Biol 12:554–555

Fagart J, Hillisch A, Huyet J, Barfacker L, Fay M, Pleiss U, Pook E, Schafer S, Rafestin-Oblin M-E, Kolkhof P (2010) A new mode of mineralocorticoid receptor antagonism by a potent and selective nonsteroidal molecule. J Biol Chem 285:29932–29940

Faresse N, Ruffieux-Daidie D, Salamin M, Gomez-Sanchez CE, Staub O (2010) Mineralocorticoid receptor degradation is promoted by Hsp90 inhibition and the ubiquitin-protein ligase CHIP. Am J Physiol Renal Physiol 299:F1462–F1472

Feldman RD, Gros R (2013) Vascular effects of aldosterone: sorting out the receptors and the ligands. Clin Exp Pharmacol Physiol 40(12):916–921

Feldman D, Funder JW, Edelman IS (1973) Evidence for a new class of corticosterone receptor in the rat kidney. Endocrinology 92:1429–1441

Fiebeler A, Schmidt F, Muller DN, Park J-K, Dechend R, Bieringer M, Shagdarsuren E, Breu V, Haller H, Luft FC (2001) Mineralocorticoid receptor affects AP-1 and nuclear factor-{{kappa}}B activation in angiotensin II-induced cardiac injury. Hypertension 37:787–793

Fischer K, Kelly SM, Watt K, Price NC, McEwan IJ (2010) Conformation of the mineralocorticoid receptor N-terminal domain: evidence for induced and stable structure. Mol Endocrinol 24:1935–1948

Funder JW (1993) Mineralocorticoids, glucocorticoids, receptors and response elements. Science 259(5098):1132–1133

Fuse H, Kitagawa H, Kato S (2000) Characterization of transactivational property and coactivator mediation of rat mineralocorticoid receptor activation function-1 (AF-1). Mol Endocrinol 14:889–899

Galigniana MD, Echeverria PC, Erlejman AG, Piwien-Pilipuk G (2010a) Role of molecular chaperones and TPR-domain proteins in the cytoplasmic transport of steroid receptors and their passage through the nuclear pore. Nucleus 1:299–308

Galigniana MD, Erlejman AG, Monte M, Gomez-Sanchez C, Piwien-Pilipuk G (2010b) The hsp90-FKBP52 complex links the mineralocorticoid receptor to motor proteins and persists bound to the receptor in early nuclear events. Mol Cell Biol 30:1285–1298

Gallo LI, Ghini AA, Pilipuk GP, Galigniana MD (2007) Differential recruitment of tetratricopeptide repeat domain immunophilins to the mineralocorticoid receptor influences both heat-shock protein 90-dependent retrotransport and hormone-dependent transcriptional activity. BioChemistry 46:14044–14057

Geerling JC, Loewy AD (2009) Aldosterone in the brain. Am J Physiol Renal Physiol 297:F559–F576

Geller DS, Farhi A, Pinkerton N, Fradley M, Moritz M, Spitzer A, Meinke G, Tsai FT, sigler PB, Lifton RP (2000) Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. Science 289:119–123

Geserick C, Meyer H-A, Barbulescu K, Haendler B (2003) Differential modulation of androgen receptor action by deoxyribonucleic acid response elements. Mol Endocrinol 17:1738–1750

Gomez-Sanchez EP, Venkataraman MT et al (1990) ICV infusion of corticosterone antagoizes ICV-aldosterone hypertension. Am J Physiol 258:E649–E653

Govindan MV, Warriar N (1998) Reconstitution of the N-terminal transcription activation function of human mineralocorticoid receptor in a defective human glucocorticoid receptor. J Biol Chem 273:24439–24447

Green S, Chambon P (1987) Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. Nature 325:75–78

Groeneweg FL, Karst H, de Kloet ER, Joëls M (2012) Mineralocorticoid and glucocorticoid receptors at the neuronal membrane, regulators of nongenomic corticosteroid signalling. Mol Cell Endocrinol 350:299–309

Grossman C, Gekle M (2012) Interaction between mineralocorticoid receptor and epidermal growth factor receptor signaling. Mol Cell Endocrinol 350:235–241

Grossman C, Ruhs S, Langenbruch L, Mildenberger S, Strätz N, Schumann K, Gekle M (2012) Nuclear shuttling precedes dimerization in mineralocorticoid receptor signaling. Chem Biol 19:742–751

Grossmann C, Krug AW, Freudinger R, Mildenberger S, Voelker K, Gekle M (2007) Aldosterone-induced EGFR expression: interaction between the human mineralocorticoid receptor and the human EGFR promoter. Am J Physiol Endocrinol Metab 292:E1790–E1800

Guo C, Ricchiuti V, Lian BQ, Yao TM, Coutinho P, Romero JR, Li J, Williams GH, Adler GK (2008) Mineralocorticoid receptor blockade reverses obesity-related changes in expression of adiponectin, peroxisome proliferator-activated receptor-gamma, and proinflammatory adipokines. Circulation 117(17):2253–2261

Hasui T, Matsunaga N, Ora T, Ohyabu N, Nishigaki N, Imura Y, Igata Y, Matsui H, Motoyaji T, Tanaka T, Habuka N, Sogabe S, Ono M, Siedem CS, Tang TP, Gauthier C, De Meese LA, Boyd SA, Fukumoto S (2011) Identification of benzoxazin-3-one derivatives as novel, potent, and selective nonsteroidal mineralocorticoid receptor antagonists. J Med Chem 54(24):8616–8631

He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM (1999) Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH2-terminal domain. J Biol Chem 274:37219–37225

Heery DM, Kalkhoven E, Hoare S, Parker MG (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736

Hirata A, Maeda N, Hiuge A, Hibuse T, Fujita K, Okada T, Kihara S, Funahashi T, Shimomura I (2009) Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice. Cardiovasc Res 84(1):164–172

Hirschberg D, Jägerbrink T, Samskog J, Gustafsson M, Stählberg M, Alvelius G, Husman B, Carlquist M, Jörnvall H, Bergman T (2004) Detection of phosphorylated peptides in proteomic analyses using microfluidic compact disk technology. Anal Chem 76:5864–5871

Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK et al (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404

Hultman ML, Krasnoperova NV, Li S, Du S, Xia C, Dietz JD, Lala DS, Welsch DJ, Hu X (2005) The ligand–dependent interaction of mineralocorticoid receptor with coactivator and corepressor peptides suggests multiple activation mechanisms. Mol Endocrinol 19:1460–1473

Huyet J, Pinon GM, Fay MR, Fagart J, Rafestin-Oblin M-E (2007) Structural basis of spirolactone recognition by the mineralocorticoid receptor. Mol Pharmacol 72:563–571

Huyet J, Pinon GM, Fay MR, Rafestin-Oblin M-E (2012) Structural determinants of ligand binding to the mineralocorticoid receptor. Mol Cell Endocrinol 350:187–195

Iniguez-Lluhi JA, Pearce D (2000) A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol Cell Biol 20:6040–6050

Jonat C, Rahmsdorf HJ, Park K-K, Cato ACB, Gebel S, Ponta H, Herrlich P (1990) Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 62:1189–1204

Juurlink DN, Mamdani MM, Lee DS, Kopp A, Austin PC, Laupacis A, Redelmeier DA (2004) Rates of hyperkalemia after publication of the randomized aldactone evaluation study. New Engl J Med 351:543–551

Karst HI, Berger S, Turiault M, Tronche F, Schütz G, Joëls M (2005) Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. Proc Natl Acad Sci U S A 102:19204–19207

Keightley MC, Curtis AJ, Chu S, Fuller PJ (1998) Structural determinants of cortisol resistance in the guinea pig glucocorticoid receptor. Endocrinology 139:2479–2485

Kino T, Jaffe H, Amin ND, Chakrabarti M, Zheng Y-L, Chrousos GP, Pant HC (2010) Cyclin-dependent kinase 5 modulates the transcriptional activity of the mineralocorticoid receptor and regulates expression of brain-derived neurotrophic factor. Mol Endocrinol 24:941–952

Klokk TI, Kurys P, Elbi C, Nagaich AK, Hendarwanto A, Slagsvold T, Chang C-Y, Hager GL, Saatcioglu F (2007) Ligand-specific dynamics of the androgen receptor at its response element in living cells. Mol Cell Biol 27:1823–1843

Kolkhof P, Borden SA (2012) Molecular pharmacology of the mineralocorticoid receptor: prospects for novel therapeutics. Mol Cell Endocrinol 350:310–317

Kolla V, Litwack G (2000) Inhibition of mineralocorticoid-mediated transcription by NF-[kappa] B. Arch Biochem Biophys 383:38–45

Lalevee S, Ferry C, Rochette-Egly C (2010) Phosphorylation control of nuclear receptors. Methods Mol Biol 647:251–266

Langley E, Zhou ZX, Wilson EM (1995) Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. J Biol Chem 270:29983–29990
Lavery DN, McEwan IJ (2005) Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem J 391:449–464
Li Y, Suino K, Daugherty J, Xu HE (2005) Structural and biochemical mechanisms for the specificity of hormone binding and coactivator assembly by mineralocorticoid receptor. Mol Cell 19:367–380
Liden J, Delaunay F, Rafter I, Gustafsson J-Ãk, Okret S (1997) A new function for the C-terminal zinc finger of the glucocorticoid receptor. J Biol Chem 272:21467–21472
Lim-Tio SS, Keightley MC, Fuller PJ (1997) Determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor. Endocrinology 138(6):2537–2543
Liu W, Wang J, Sauter NK, Pearce D (1995) Steroid receptor heterodimerization demonstrated in vitro and in vivo. Proc Natl Acad Sci U S A 92:12480–12484
Lombes M, Binart N, Oblin ME, Joulin V, Baulieu EE (1993) Characterization of the interaction of the human mineralocorticosteroid receptor with hormone response elements. Biochem J 292(Pt. 2):577–583
Loven MA, Likhite VS, Choi I, Nardulli AM (2001) Estrogen response elements alter coactivator recruitment through allosteric modulation of estrogen receptor Î2 conformation. J Biol Chem 276:45282–45288
Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505
Martinez ED, Pattabiraman N, Danielsen M (2005) Analysis of the hormone-binding domain of steroid receptors using chimeras generated by homologous recombination. Exp Cell Res 308:320–333
Marzolla V, Armani A, Zennaro M-C, Cinti F, Mammi C, Fabbri A, Rosano GMC, Caprio M (2012) The role of the mineralocorticoid receptor in adipocyte biology and fat metabolism. Mol Cell Endocrinol 350:281–288
Massaad C, Lombès M, Aggerbeck M, Rafestin-Oblin M-E, Barouki R (1997) Cell-specific, promoter-dependent mineralocorticoid agonist activity of spironolactone. Mol Pharmacol 51:285–292
McCurley A, Jaffe IZ (2012) Mineralocorticoid receptors in vascular function and disease. Mol Cell Endocrinol 350:256–265
McEneaney V, Dooley R, Yusef YR, Keating N, Quinn U, Harvey BJ, Thomas W (2010) Protein kinase D1 modulates aldosterone-induced ENaC activity in a renal cortical collecting duct cell line. Mol Cell Endocrinol 325:8–17
McEwan IJ, Lavery D, Fischer K, Watt K (2007) Natural disordered sequences in the amino terminal domain of nuclear receptors: lessons from the androgen and glucocorticoid receptors. Nucl Recept Signaling [Electronic Resource] E-J NURSA 5:e001
McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Inostroza J, Torchia J, Nolte RT, Assa-Munt N, Milburn MV, Glass CK, Rosenfeld MG (1998) Determinants of co-activator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Develop 12:3357–3368
Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324:407–410
Mètivier R, Stark A, Flouriot G, Hübner MR, Brand H, Penot G, Manu D, Denger S, Reid G, Kos M, Russell RB, Kah O, Pakdel F, Gannon F (2002) A dynamic structural model for estrogen receptor-a activation by ligands, emphasizing the role of interactions between distant A and E domains. Mol Cell 10:1019–1032
Meyers MJ, Arhancet GB, Hockerman SL, Chen X, Long SA, Mahoney MW, Rico JR, Garland DJ, Blinn JR, Collins JT, Yang S, Huang HC, McGee KF, Wendling JM, Dietz JD, Payne MA, Homer BL, Heron MI, Reitz DB, Hu X (2010) Discovery of (3 S,3aR)-2-(3-chloro-4-cyanophenyl)-3-cyclopentyl-3,3a,4,5-tetrahydro-2H-benzo[g]indazole-7-carboxylic acid (PF-3882845), an orally efficacious mineralocorticoid receptor (MR) antagonist for hypertension and nephropathy. J Med Chem 53(16):5979–6002

Mihailidou AS (2006) Nongenomic actions of aldosterone: physiological or pathophysiological role? Steroids 71:277–280

Murai-Takeda A, Shibata H, Kurihara I, Kobayashi S, Yokota K, Suda N, Mitsuishi Y, Jo R, Kitagawa H, Kato S, Saruta T, Itoh H (2010) NF–YC functions as a corepressor of agonist-bound mineralocorticoid receptor. J Biol Chem 285:8084–8093

Nakshatri H, Bhat-Nakshatri P, Currie RA (1996) Subunit association and DNA binding activity of the heterotrimeric transcription factor NF–Y is regulated by cellular redox. J Biol Chem 271:28784–28791

Nishi M (2010) Imaging of transcription factor trafficking in living cells: lessons from corticosteroid receptor dynamics. Methods Mol Biol 647:199–212

Nordeen SK, Bona BJ, Beck CA, Edwards DP, Borror KC, DeFranco DB (1995) The two faces of a steroid antagonist: when an antagonist isn't. Steroids 60:97–104

O'Malley BW (2007) Coregulators: from whence came these "master genes". Mol Endocrinol 21:1009–1013

Ou X-M, Storring JM, Kushwaha N, Albert PR (2001) Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene. J Biol Chem 276:14299–14307

Pascual-Le Tallec L, Kirsh O, Lecomte M-C, Viengchareun S, Zennaro M-C, Dejean A, Lombes M (2003) Protein inhibitor of activated signal transducer and activator of transcription 1 interacts with the N-terminal domain of mineralocorticoid receptor and represses its transcriptional activity: Implication of small ubiquitin-related modifier 1 modification. Mol Endocrinol 17:2529–2542

Pascual-Le Tallec L, Simone F, Viengchareun S, Meduri G, Thirman MJ, Lombes M (2005) The elongation factor ELL (eleven-nineteen lysine-rich leukemia) is a selective coregulator for steroid receptor functions. Mol Endocrinol 19:1158–1169

Paul A, Garcia YA, Zierer B, Patwardhan C, Gutierrez O, Hildenbrand Z, Harris DC, Balsiger HA, Sivils JC, Johnson JL, Buchner J, Chadli A, Cox MB (2014) The cochaperone SGTA (small glutamine-rich tetratricopeptide repeat-containing protein alpha) demonstrates regulatory specificity for the androgen, glucocorticoid and progesterone receptors. J Biol Chem 289:15297–15308

Pearce D (1994) A mechanistic basis for distinct mineralocorticoid and glucocorticoid receptor transcriptional specificities. Steroids 59:153–159

Pearce D, Yamamoto KR (1993) Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science 259:1161–1165

Pippal J, Fuller P (2008) Structure-function relationships in the mineralocorticoid receptor. J Mol Endocrinol. doi:10.1677/JME-08-0093

Pippal JB, Yao Y, Rogerson FM, Fuller PJ (2009) Structural and functional characterization of the interdomain interaction in the mineralocorticoid receptor. Mol Endocrinol 23:1360–1370

Pippal JB, Cheung CMI, Yao Y-Z, Brennan FE, Fuller PJ (2011) Characterization of the zebrafish (Danio rerio) mineralocorticoid receptor. Mol Cell Endocrinol 332:58–66

Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J (1999) The randomized aldactone evaluation study (1999) The effect of spironolactone on morbidity and mortality in patients with severe heart failure. New Engl J Med 341:709–717

Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M, Eplerenone post-acute myocardial infarction heart failure, and I. Survival study (2003) Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. New Engl J Med 348:1309–1321

Pitt B, Kober L, Ponikowski P, Gheorghiade M, Filippatos G, Krum H, Nowack C, Kolkhof P, Kim SY, Zannad F (2013) Safety and tolerability of the novel non-steroidal mineralocorticoid receptor antagonist BAY 94-8862 in patients with chronic heart failure and mild or moderate chronic kidney disease: a randomized, double-blind trial. Eur Heart J 34(31):2453–2463

Poukka H, Karvonen U, Jänne OA, Palvimo JJ (2000a) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci U S A 97:14145–14150

Poukka H, Karvonen U, Janne OA, Palvimo JJ (2000b) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci U S A 97:14145–14150

Pratt WB, Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18:306–360

Quigley CA, Tan JA, He B, Zhou ZX, Mebarki F, Morel Y, Forest MG, Chatelain P, Ritzèn EM, French FS, Wilson EM (2004) Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the $NH_{(2)}$- and carboxyl-terminal interaction. Mech Ageing Dev 125:683–695

Rafestin-Oblin ME, Couette B, Radanyi C, Lombes M, Baulieu EE (1989) Mineralocorticosteroid receptor of the chick intestine. Oligomeric structure and transformation. J Biol Chem 264:9304–9309

Rafestin-Oblin ME, Souque A, Bocchi B, Pinon G, Fagart J, Vanderwalle A (2003) The severe form of hypertension caused by the activating S810L mutation in the mineralocorticoid receptor is cortisone related. Endocrinology 144:528–533

Ray A, Prefontaine KE (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. Proc Natl Acad Sci U S A 91:752–756

Rickard AJ, Morgan J, Tesch G, Funder JW, Fuller PJ, Young MJ (2009) Deletion of mineralocorticoid receptors from macrophages protects against DOC/salt-induced cardiac fibrosis and increased blood pressure. Hypertension 54:537–743

Rickard AJ, Morgan J, Bienvenu LA, Fletcher EK, Cranston GA, Shen JZ, Reichelt ME, Delbridge LM, Young MJ (2012) Cardiomyocyte mineralocorticoid receptors are essential for deoxycorticosterone/salt-mediated inflammation and cardiac fibrosis. Hypertension 60(6):1443–1450

Robin-Jagerschmidt C, Wurtz J-M, Guillot B, Gofflo D, Benhamou B, Vergezac A, Ossart C, Moras D, Phililbert D (2000) Residues in the ligand binding domain that confer progestin or glucocorticoid specificity and modulate the receptor transactivation capacity. Mol Endocrinol 14:1028–1037

Rogerson FM, Fuller PJ (2003) Interdomain interactions in the mineralocorticoid receptor. Mol Cell Endocrinol 200:45–55

Rogerson FM, Dimopoulos N, Sluka P, Chu S, Curtis AJ, Fuller PJ (1999) Structural determinants of aldosterone binding selectivity in the mineralocorticoid receptor. J Biol Chem 274:36305–36311

Rogerson FM, Yao Y-Z, Smith BJ, Dimopoulos N, Fuller PJ (2003) Determinants of spironolactone binding specificity in the mineralocorticoid receptor. J Mol Endocrinol 31:573–582

Rogerson FM, Yao Y-Z, Smith BJ, Fuller PJ (2004) Differences in the determinants of eplerenone, spironolactone and aldosterone binding to the mineralocorticoid receptor. Clin Exp Pharmacol Physiol 31:704–709

Rogerson FM, Yao Y-Z, Elsass RE, Dimopoulos N, Smith BJ, Fuller PJ (2007) A critical region in the mineralocorticoid receptor for aldosterone binding and activation by cortisol: evidence for a common mechanism governing ligand binding specificity in steroid hormone receptors. Mol Endocrinol 21:817–828

Rogerson FM, Yao YZ, Young MJ, Fuller PJ (2014) Identification and characterization of a ligand-selective mineralocorticoid receptor coactivator. FASEB J. doi:10.1096/fj.13-242479. [Epub ahead of print]

Rupprecht R, Arriza JL, Spengler D, Reul JM, Evans RM, Holsboer F, Damm K (1993) Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. Mol Endocrinol 7(4):597–603

Sato A, Funder JW (1996) High glucose stimulates aldosterone-induced hypertrophy via type I mineralocorticoid receptors in neonatal rat cardiomyocytes. Endocrinol 137:4145–4153

Savory JGA, Prefontaine GG, Lamprecht C, Liao M, Walther RF, Lefebvre YA, Hache RJG (2001) Glucocorticoid receptor homodimers and glucocorticoid mineralocorticoid receptor heterodimers form in the cytoplasm through alternative dimerization interfaces. Mol Cell Biol 21:781–793

Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AAK, Miner JN, Diamond MI (2005) The structural basis of androgen receptor activation: intramolecular and intermolecular amino–carboxy interactions. Proc Natl Acad Sci U S A 102:9802–9807

Seeler J-S, Dejean A (2003) Nuclear and unclear functions of SUMO. Nat Rev Mol Cell Biol 4:690–699

Shavit L, Lifschitz MD, Epstein M (2012) Aldosterone blockade and the mineralocorticoid receptor in the management of chronic kidney disease: current concepts and emerging treatment paradigms. Kidney Int 81(10):955–968

Shibata S, Rinehart J, Zhang J, Moeckel G, Castañeda-Bueno M, Stiegler AL, Boggon TJ, Gamba G, Lifton RP (2013) Mineralocorticoid receptor phosphorylation regulates ligand binding and renal response to volume depletion and hyperkalemia. Cell Metab 18(5):660–671

Stow LR, Gumz ML, Lynch IJ, Greenlee MM, Rudin A, Cain BD, Wingo CS (2009) Aldosterone modulates steroid receptor binding to the endothelin-1 Gene (edn1). J Biol Chem 284:30087–30096

Sturm A, Bury N, Dengreville L, Fagart J, Flouriot G, Rafestin-Oblin ME, Prunet P (2005) 11-deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146:47–55

Tan J-A, Hall SH, Hamil KG, Grossman G, Petrusz P, Liao J, Shuai K, French FS (2000) Protein inhibitor of activated STAT-1 (signal transducer and activator of transcription-1) is a nuclear receptor coregulator expressed in human testis. Mol Endocrinol 14:14–26

Tetel MJ, Giangrande PH, Leonhardt SA, McDonnell DP, Edwards DP (1999) Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. Mol Endocrinol 13:910–924

Thompson J, Saatcioglu F, Janne OA, Palvimo JJ (2001) Disrupted amino- and carboxyl–terminal interactions of the androgen receptor are linked to androgen insensitivity. Mol Endocrinol 15:923–935

Tian S, Poukka H, Palvimo JJ, Jänne OA (2002) Small ubiquitin-related modifier-1 (SUMO-1) modification of the glucocorticoid receptor. Biochem J 367:907–911

Tirard M, Jasbinsek J, Almeida OF, Michaelidis TM (2004) The manifold actions of the protein inhibitor of activated STAT proteins on the transcriptional activity of mineralocorticoid and glucocorticoid receptors in neural cells. J Mol Endocrinol 32:825–841

Tirard M, Almeida OFX, Hutzler P, Melchior F, Michaelidis TM (2007) Sumoylation and proteasomal activity determine the transactivation properties of the mineralocorticoid receptor. Mol Cell Endocrinol 268:20–29

Trapp T, Rupprecht R, Castrén M, Reul JMHM, Holsboer F (1994) Heterodimerization between mineralocorticoid and glucocorticoid receptor: a new principle of glucocorticoid action in the CNS. Neuron 13:1457–1462

Tsuji M (2013) Local motifs involved in the canonical structure of the ligand-binding domain in the nuclear receptor superfamily. J Struct Biol. doi:org/10.1016/jjsb.2013.12.007

Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes M (2007) The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. Nucl Recept Signaling [Electronic Resource] E-J NURSA 5:e012

Vivat V, Gofflo D, Wurtz J-M, Bourguet W, Philibert D, Gronemeyer H (1997) Sequences in the ligand-binding domains of the human androgen and progesterone receptors which determine their distinct ligand identities. J Mol Endocrinol 18:147–160

Vlassi M, Brauns K, Andrade-Nararro MA (2013) Short tandem repeats in the inhibitory domain of the mineralocorticoid receptor: prediction of a b-solenoid structure. BMC Struct Biol 13:17. doi:10.1186/1472-6807-13-17

Walther RF, Atlas E, Carrigan A, Rouleau Y, Edgecombe A, Visentin L, Lamprecht C, Addicks GC, Hache RJG, Lefebvre YA (2005) A serine/threonine-rich motif is one of three nuclear localization signals that determine unidirectional transport of the mineralocorticoid receptor to the nucleus. J Biol Chem 280:17549–17561

Wang Q, Anzick S, Richter WF, Meltzer P, Simons SS Jr (2004) Modulation of transcriptional sensitivity of mineralocorticoid and estrogen receptors. J Steroid Biochem Mol Biol 91:197–210

Wochnik GM, Räegg J, Abel GA, Schmidt U, Holsboer F, Rein T (2005) FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J Biol Chem 280:4609–4616

Yang J, Fuller PJ (2012) Interactions of the mineralocorticoid receptor—within and without, 2012. Mol Cell Endocrinol 350:196–205

Yang J, Young MJ (2009) The mineralocorticoid receptor and its coregulators. J Mol Endocrinol 43:53–64

Yang J, Chang C-Y, Safi R, Morgan J, McDonnell DP, Fuller PJ, Clyne CD, Young MJ (2011) Identification of ligand-selective peptide antagonists of the mineralocorticoid receptor using phage display. Mol Endocrinol 25:32–43

Yang J, Fuller PJ, Morgan J, Shibata H, McDonnell DP, Clyne CD, Young MJ (2014) Use of phage display to identify novel mineralocorticoid receptor-interacting proteins. Mol Endocrinol. doi:10.1210/me20141101. [Epub ahead of print]

Yokota K, Shibata H, Kurihara I, Kobayashi S, Suda N, Murai-Takeda A, Saito I, Kitagawa H, Kato S, Saruta T, Itoh H (2007) Coactivation of the N-terminal transactivation of mineralocorticoid receptor by Ubc9. J Biol Chem 282:1998–2010

Young MJ, Rickard AJ (2012) Mechanisms of mineralocorticoid salt-induced hypertension and cardiac fibrosis. Mol Cell Endocrinol 350:248–255

Zannad F, McMurray JJV, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, Vincent J, Pocock SJ, Pitt B (2010) Eplerenone in patients with systolic heart failure and mild symptoms. New Engl J Med 364:11–21

Zhang J, Geller DS (2008) Helix 3-helix 5 interactions in steroid hormone receptor function. J Steroid Biochem Mol Biol 109(3–5):279–285

Zhang J, Simisky J, Tsai FT, Geller DS (2005) A critical role of helix 30helix 5 interaction in steroid hormone receptor function. Proc Natl Acad Sci U S A 102:2707–2712

Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM (1995) Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 9:208–218

Glucocorticoid Receptor Structure and Function

Raj Kumar and Iain J. McEwan

1 Introduction

Glucocorticoids are essential hormones for life that are synthesized by the adrenal cortex in response to stress and their secretion controlled by the hypothalamic-pituitary-adrenal axis (Oakley and Cidlowski 2013). Acting on nearly every tissue and organ, the actions of glucocorticoids in modulating a large number of biological processes including development, metabolic, cardiovascular, immune, stress responses, and behavioral functions is well established (Barnes 1998; Oakley and Cidlowski 2013). Glucocorticoids and their steroid analogs are also among the most potent and effective agents used clinically for treating inflammation and autoimmune diseases including asthma, allergy, and rheumatoid arthritis as well as in the treatment of certain cancers such as leukemia and lymphoma (Beck et al. 2009; Ahmad and Kumar 2011; Inaba and Pui 2010; Thompson and Johnson 2003; Frankfurt and Rosen 2004). However, long term therapeutic dosages of glucocorticoids induce a range of debilitating side effects such as diabetes, osteoporosis, skin atrophy, bone loss, growth retardation and suppression of the hypothalamic-pituitary-adrenal axis (Oakley and Cidlowski 2013; Beck et al. 2009) and thereby limit their clinical uses. Therefore, the discovery and development of novel synthetic glucocorticoid steroids that can retain their beneficial therapeutic effects, but reduce adverse side effects remain one of the major challenges for scientists and clinicians, and subject of intense pharmaceutical efforts.

The physiological and pharmacological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR/NR3C1). The GR belongs to the nuclear

R. Kumar (✉)
Department of Basic Sciences, The Commonwealth Medical College,
525 Pine Street, Scranton, PA 18509, USA
e-mail: rkumar@tcmc.edu

I. J. McEwan
School of Medical Sciences, Programme of Cell, Developmental and Cancer Biology,
University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, Scotland, UK
e-mail: iain.mcewan@abdn.ac.uk

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_3

receptor superfamily of the intracellular transcription factors (Evans 1995; Beato et al. 1996; Reichardt and Schutz 1998). Like most nuclear receptors, GR is a modular protein consisting of three major functional domains: an N-terminal domain (NTD), a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD). In addition to its role in ligand recognition, the LBD contains a ligand-dependent activation function (AF2) that is tightly regulated by hormone binding (Kumar and Thompson 2005). Another activation function (AF1) located within the NTD is constitutively active and in the absence of LBD is capable of acting in a ligand-independent manner. However, recent studies have suggested that in the context of the full-length receptor, the AF1 activity is dependent on steroid/hormone binding. According to the classical mechanism of action (Fig. 1), in the absence of any ligand, GR is localized in the cytoplasm associated with chaperone proteins including hsp90, hsp70, hsp40, p23, and several other proteins (Pratt and

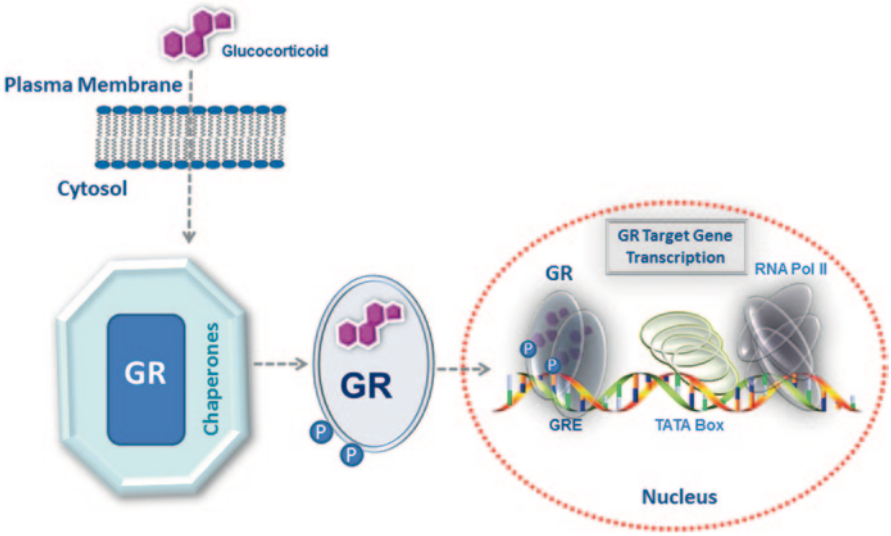


Fig. 1 Classical action of the glucocorticoid signaling mediated by the GR**.** Unliganded receptor is located in the cytosol associated with several heat shock and other chaperone proteins including HSP90, HSP70, HSP40, P23, and FKBPs (shown by light blue shades around GR). Ligand binding leads to conformational alterations in the GR, and by doing so GR dissociates from these associated proteins, and ligand bound GR is free to translocate to the nucleus. This process appears to be phosphorylation dependent. Once in the nucleus, GR dimerizes and binds to site-specific DNA binding sequences and interacts with several other coregulatory proteins including coactivators and proteins from the basal transcription machinery such as SRCs, CBP/p300, DRIP/TRAP, TBP, GRIP1, and several others (shown by different shapes and shades) in the nucleus, and subsequently leading to transcriptional regulation. The cofactor(s) bound are determined by their levels in particular cell types and by the state of the folded structure of the GR. The complex alters local chromatin structure, e.g., by catalysing histone acetylation or deacetylation, and affects the stabilization of the transcription pre-initiation complex [TATA-box-binding protein (TBP)–TBP-associated factors (TAFs)–RNA polymerase II (RNA Pol II. The activity of kinases and phosphatases regulating signaling pathways also contribute to this process by altering the state of phosphorylation of both receptor and cofactors (not shown)

Toft 1997). Once ligand-bound, the GR undergoes conformational rearrangement that allows its dissociation from chaperone proteins, leading to dimerization and translocation of the receptor into the nucleus. While in the nucleus, GR binds to its site-specific DNA sequences termed as glucocorticoid response elements (GREs) to either activate or repress transcription of the target genes in a promoter dependent manner. Alternatively, GR can also interact with other transcriptional factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) to repress their gene activation for the anti-inflammatory and immunosuppressive activities of glucocorticoids (De Bosscher et al. 2003; Kumar and Thompson 1999; Beck et al. 2009).

The molecular mechanism of ligand-dependent regulation of GR activity has been illustrated by the crystal structure of ligand-bound LBD which highlights the importance of the conformational dynamics of helix-12 in forming the AF2 surface on the LBD allowing interactions with LxxLL motifs found in coregulatory proteins, including coactivators and/or corepressors (Bledsoe et al. 2002; Rosen and Miner 2005**)**. This phenomenon has extensively been exploited for the evolution of small molecule selective glucocorticoid receptor modulators driven by the demand of lowering the unwanted side effects, while keeping the beneficial anti-inflammatory effects (He et al. 2014; Rosen and Miner 2005). Potency and efficacy are two key pharmacokinetic parameters of glucocorticoids and the observed mechanisms of action based on the LBD structure to increase potency and efficacy of several newly designed glucocorticoids (Fig. 2) have served as starting leads for the development of novel therapeutics for the treatment of inflammatory diseases (Simons 2008; Simons 2010; Frey et al. 2004). Potency is an important aspect of this evolution as many undesirable side effects are associated with the use of high-dose glucocorticoids, which can be minimized by highly potent glucocorticoids that may achieve similar treatment effects at lower doses.

Given its biological and pharmaceutical importance, there has been enormous interest in elucidating the 3-dimensional structure of full length GR. Since GR is highly homologous to mineralocorticoid (MR/NR3C2), androgen (AR/NR3C4), and progesterone (PR/NR3C3) receptors, when determined the structure should serve as a model for understanding the roles of ligand binding, GRE binding, coactivator recruitment, and receptor dimerization among others in the signaling pathways mediated by these steroid receptors. In this article, we discuss the current understanding of the available structural information of the GR with a special emphasis on the importance of conformational dynamics that plays a critical role in

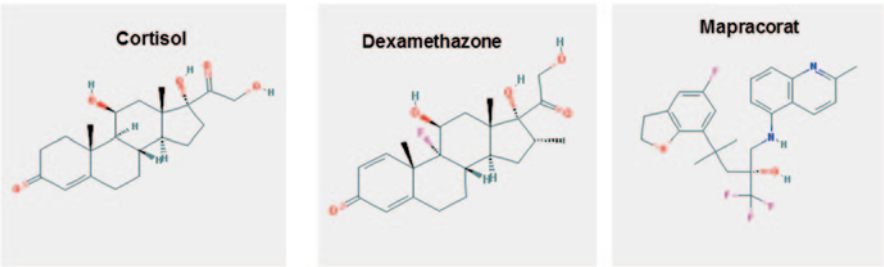


Fig. 2 Structure of some glucocorticoid molecules. (source: PubChem structure search)

the receptor action, and how this information can be used in structure-based drug design, which could serve as lead compounds for the development of novel therapeutics for the treatment of inflammatory diseases.

2 Glucocorticoid Receptor Isoform Expressions and Cell/Tissue Specific Transcriptional Activity of Glucocorticoid-Responsive Genes

The human GR protein is encoded by exons 2–9 located on chromosome 5, and due to alternative splicing gives rise to several GR protein subtypes (Fig. 3). The GR NTD is encoded primarily by exon 2, the DBD is encoded by exons 3 and 4, and the hinge region and LBD are encoded by exons 5–9 (Lu and Cidlowski 2005).

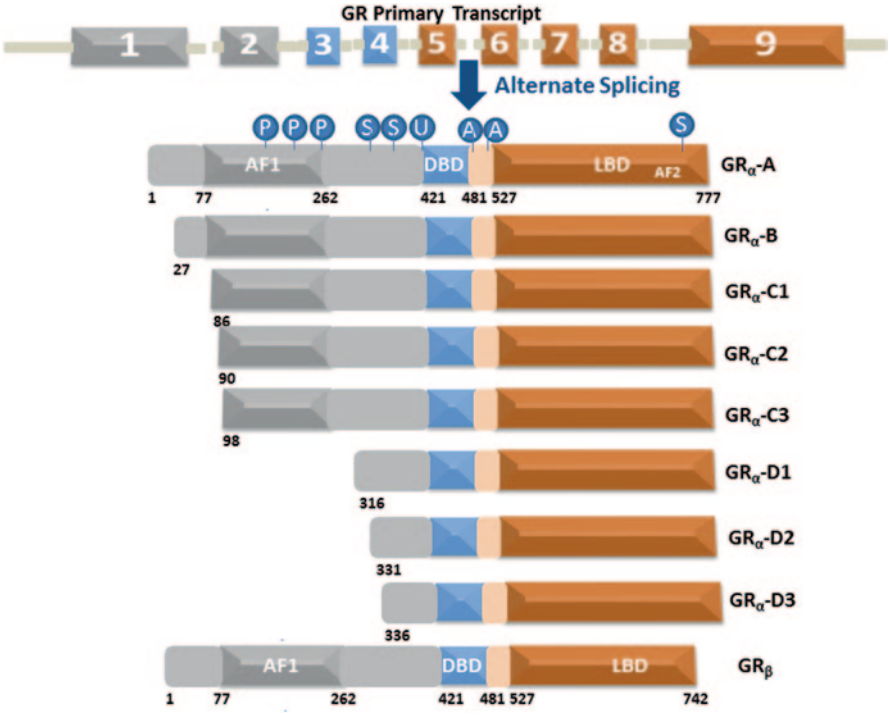


Fig. 3 The GR undergoes alternative processing to yield multiple functionally distinct subtypes. Human GR contains 9 exons with the protein coding region formed by 2–9. The GRα undergoes alternative translation initiation in exon 2, generating GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3 isoforms with truncated NTD. GR is a modular protein containing NTD, DBD, LBD, and a flexible "hinge region" separating the DBD and the LBD. The NTD and LBD possess transcriptional activation function regions AF1 and AF2, respectively. GR undergoes multiple posttranslational modifications including phosphorylation (*P*), SUMOylation (*S*), ubiquitination (*U*) and acetylation (*A*)

Alternative splicing in exon 9 near the end of the GR primary transcript generates two receptor isoforms, termed GRα and GRβ (Oakley and Cidlowski 2011). GRβ is prevalent in many cells and tissues but generally is found at lower levels than GRα. Elevated levels of GRβ have been associated with glucocorticoid resistance in a variety of inflammatory diseases. The GRα-A generated from the first translation initiation codon is often termed as the classic full-length receptor protein consisting of 777 amino acids (Yudt and Cidlowski 2001; Oakley and Cidlowski 2011). Other GRα isoforms resulting from alternative RNA splicing and translation initiation of the GRα transcript that possess additional unique properties may differentially regulate GR target genes depending on their selective and relative expression in specific tissues/cells (Oakley and Cidlowski 2011).

Each individual GR isoform originating from alternative processing of the GR gene is subject to a variety of post-translational modifications that further modulate function and expand the repertoire of receptor subtypes available for glucocorticoid signaling. Normally these splice variants have shortened NTD and unhindered DBD and LBD sequences that makes them capable of producing DBD/DNA and ligand/LBD binding with similar affinity as that of GRα-A. Since they all possess intact DBD and LBD including the hinge region, it is logical to argue that in the absence of ligand binding all the receptor isoforms should also possess similar affinity for HSP90 and other chaperone machinery proteins and nuclear translocation signals resulting into their localization to the cytosols. However, recent studies have shown that they display differences in their cytosolic and nuclear distribution (Lu and Cidlowski 2005; Oakley and Cidlowski 2011).

This raises an important question: does NTD sequences play role in nuclear translocation, nuclear export and/or cytoplasmic retention of the receptor? This is an important question and answering it may not only play a critical role in defining the tissue/cell specific effects of GR isoforms, but may also provide a better understanding of target-specific gene regulation leading to the design of new therapeutic drug. The GR*α* translational isoforms display a similar affinity for glucocorticoids and a similar capacity to interact with GREs following ligand activation; however, they show marked differences in other properties. It has been reported that the human GR isoforms that lack AF1 sequences (e.g., GRα-D) are virtually inactive in their ability to transactivate glucocorticoid-responsive genes (Lu and Cidlowski 2005; Oakley and Cidlowski 2011). On the other hand, isoforms that lack amino acid sequences (GR-C isoforms; C1, C2, and C3) prior to or in the early part of AF1 produce differing GR activities in a cell-dependent manner, suggesting that NTD sequences around AF1 may play roles in regulating GR activity (Kumar and Thompson 2010; Kumar and Thompson 2012). Recent evidence suggests that isoform GRα-C3, which lacks amino acids located on the N-terminal flanking sequences of AF1, appears to be most active in displaying the transcriptional activity of glucocorticoid-responsive genes than the other hGRα isoforms, suggesting that this flanking region may be inhibitory to GR activity (Kumar and Thompson 2010; Kumar and Thompson 2012).

It has been hypothesized that the increased activity in the N-terminal truncated GR may be due to its effects on AF1 conformation and subsequent coactivator interaction (Lu and Cidlowski 2005; Kumar and Thompson 2012). In a recent study, it was found that the removal of all flanking sequences around AF1 affects

its structure such that otherwise intrinsically disordered AF1 acquires more helical structure when in direct proximity of the DBD (Kumar and Thompson 2010). The results from this study show that placing AF1 immediately upstream from the DBD leads to acquisition of an ordered conformation in AF1. Furthermore, when proximity to the DBD caused AF1 folding, this region bound CREB binding protein (CBP/p300), but not steroid receptor coactivator-1 (SRC-1), suggesting that specific region adopts differing folded configurations to bind various factors. Also, deletion of NTD sequences flanking both sides of AF1 increased GRE-mediated AF1 activity, suggesting that these flanking sequences play an inhibitory role in regulating GR AF1 structure and activity. Together these results provide a mechanism explaining why certain N-terminally truncated GR isoforms and possibly other steroid hormone receptors differ in their actvities.

Previous studies have shown that the binding of a two-domain GR fragment (consisting of entire NTD through the end DBD) to a consensus GRE sequences leads to imposition of higher secondary/tertiary structure in the NTD/AF1 whereas the AF1 bound directly to the DBD had structure and did not gain any further secondary/tertiary structure when the DBD was bound to a GRE (Kumar et al. 1999; Kumar and Thompson 2003). It therefore appears that in the full two-domain GR protein, relatively more energy is required to fold AF1 than in the construct wherein AF1 is directly hooked to DBD. This energy presumably is provided by DBD binding to its GRE. Though preliminary in nature, this maybe an important phenomenon and a detailed analysis of these observations could in fact provide a rationale for promoter specific GR activity. Yamamoto and coworkers have shown the differential effects of binding various GRE sequences on the functions of the GR that correlated with slight variations in DBD structure and lent strong evidence supporting the suggestion that a DNA sequence-specific allosteric coupling between the DBD and AF1 plays a critical role in GR activity (Meijsing et al. 2009).

3 Structure of the Glucocorticoid Receptor and Control of the Glucocorticoid-Mediated Receptor Actions

Although structures of the independently expressed recombinant proteins of the DBD and LBD of the GR have provided major advances in our understanding of the molecular basis of transcription regulation by GR (Fig. 4), a complete description of structure-function relationships and mechanism of action of GR necessitates structural studies of the full-length receptor protein. However, as yet the structure of full length GR has been elusive as has been the case with all other members of the steroid receptor subfamily of proteins. The three-dimensional structures of the DBD and LBD, expressed as independent polypeptides, have been solved, and show overall folds that represent globular proteins with natively-ordered conformations (Luisi et al. 1991; Bledsoe et al. 2002). Solution NMR structure of the DBD was the first structure determined for any region of the GR followed by crystal structure of DBD complexed with DNA sequences, showing that DBD contains two perpendicularly oriented α-helices. One of these helices is responsible for site-specific DNA recognition at the GREs

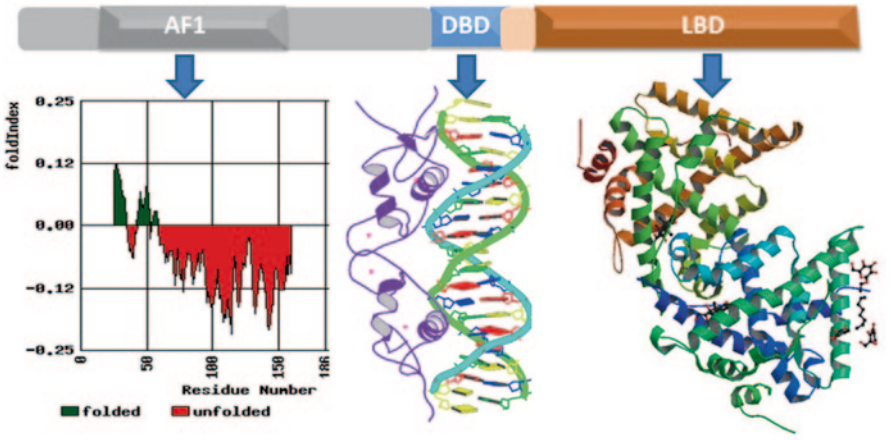


Fig. 4 Structure of the GR protein. Upper panel shows topological diagram of GR. Lower middle panel shows 3-dimensional structure GR DBD in complex with GRE (Structure 1GLU from PDB protein databank; Luisi et al. 1991). Lower right panel shows 3-dimensional structure of ligand-bound GR (PDB structures 1 M2Z; Bledsoe et al. 2002). Lower left hand panel shows Index of the probability of AF1 sequences for the propensity to fold. The red color shows the probability of unfolded and green color for the probability of folded regions. Fold Index was performed (http://bip.weizmann.ac.il/fldbin/findex) as described (Prilusky et al. 2005)

sequences (Härd et al. 1990; Luisi et al. 1991) . Consistent with the known cooperative binding kinetics of the DBD to the GRE, the structure shows a DBD dimer on the palindromic GRE. As a consequence of the unnatural four base-pair spacer, however, the two monomers in the dimer show a differential binding to the GRE. When in solution DBD structure was determined by NMR, a significant conformational flexibility in the parts of the second zinc finger was observed, which was well resolved in the crystal, perhaps due to stabilization upon formation of the dimeric DBD-DNA complex (Hard et al. 1990; Meijsing et al. 2009). Further, molecular dynamics simulation and free energy perturbation studies suggest that the binding of the DBD to the GRE distorted conformations for bases at positions 5 and 6, propagation of which through the DNA may facilitate cooperative binding of another monomer at half-site (Baumann et al. 1993; Meijsing et al. 2009; Eriksson and Nilsson 1995).

Recent structural analyses have highlighted the allosteric properties of DNA binding suggesting that receptor binding sites associated with glucocorticoid-regulated genes influence the composition of coregulatory complexes recruited by the GR (Watson et al. 2013; Meijsing et al. 2009). The binding of the GR-DBD to different response elements was found to adopt different conformations depending on the DNA architecture (Meijsing et al. 2009). The altered structure of the DBD depended on the base sequence of the response element to which it was bound, and demonstrated functional consequences as to the strength and selectivity in gene induction (Meijsing et al. 2009; Schiller et al. 2014). These structural studies reveal the conformational dynamics imparted by DNA recognition/binding and highlight the potential regulatory role of different DNA sequences or binding coregulators, which could lead to cell or promoter selective GR function.

The crystal structure of the GR LBD consists of 12 helices that fold overall into a globular structure consisting of three sets of helices that form the sides and top of the globule, making a central pocket, the ligand-binding site where the steroid molecule binds (Bledsoe et al. 2002). The helix-12 contains the sequence for the important AF2 function, the site upon which binding of coregulatory proteins depends. Helix-12 changes position upon ligand binding, flipping from an "open" position to one closed over the bound ligand resulting into agonist-bound LBD presenting a surface favorable for binding the coactivators whereas some ligands that act as antagonists appear to cause helix-12 conformation to close in a position that creates an unfavorable surface for coactivator binding (Carson et al. 2014; Schoch et al. 2010; Tao et al. 2008). In spite of having lot of similarities with the crystal structures of several other nuclear receptors, there are some unique features such as formation of a dimer interface, which is different to that seen in the ER structure (Bledsoe et al. 2002; Brzozowski et al. 1997). Importantly, mutations that can disrupt this dimerization results into the loss of glucocorticoid-mediated GR transactivation, but not transrepression activity (He et al. 2014; Bledsoe et al. 2002).

From these crystal structures, it was initially thought that both the DBD and LBD have a stable, well-ordered globular structure; however, as we learned more and more about them, it became clear that each is more structurally dynamic than once believed. The LBD responds to various ligands in a gene-selective fashion, implying that such "selective modulators" affect LBD/AF2 structure (De Bosscher 2010). Availability of LBD crystal structure and further understanding of the conformational dynamics of the structural model of helix-12 folding/unfolding with agonist and antagonist ligands explains some data that has extensively been used to design small molecule selective GR modulators for clinical use. However, still unexplained is the fact that some antagonists act as agonists in specific tissues/cells. Compared to DBD and LBD, structurally the NTD is least explored even though AF1 located in the NTD was established long before AF2 to be the major transactivation region and that much of the GR's transcriptional activity depends on this domain (Miesfeld et al. 1987; Hollenberg et al. 1987; Dahlman-Wright et al. 1994; Dahlman-Wright et al. 1995).

The major obstacle in determining the structure of NTD has been due to the fact that NTD exists in an intrinsically disordered conformation or as an ensemble of conformers that lacks any significant stable secondary/tertiary structures (Dahlman-Wright et al. 1995; Baskakov et al. 1999). This phenomenon has been observed for many transcription factors including the steroid receptors (Kumar and McEwan 2012). In recent years, it has become quite evident that intrinsically disordered proteins are highly dynamic capable of adopting structural flexibilities that can be manipulated for the interaction with the target molecules such that in multi-protein assembly specific components of protein sub-sets could either be included or excluded in a very rapid fashion. The flexible AF1 domain is ideally suited to provide such modulated surfaces in the GR, which requires assembly of specific coregulatory proteins to regulate target gene expression in a promoter and cell/tissue specific manner. Available data strongly suggest that conditional folding/stabilization of the GR NTD/AF1 is the key for these interactions and subsequent transcriptional activity (Henriksson et al. 1997; Ford et al. 1997; Almlof et al. 1998; Warnmark et al. 2000; Kumar et al. 2001; Khan et al. 2012;

Khan et al. 2011a, b). How NTD/AF1 folds and what kind of functional folded conformation it adopts are open questions, actively being pursued. The data indicate that such a folded conformation could be caused by the presence specific solute molecules such as osmolytes, which are present in cells under physiological conditions (Kumar et al. 1999; Kumar et al. 2001; Kumar et al. 2007; Khan et al. 2011a).

These studies have shown that when GR AF1 is incubated in increasing concentrations of osmolytes, the protein cooperatively folds into a compact, monomeric structure such that AF1 selectively binds TATA box-binding protein (TBP), CREB-binding protein (CBP) and a member of the steroid receptor coactivator-1 (SRC-1) family of proteins (Kumar et al. 2001; Kumar et al. 2007; Khan et al. 2011a). Another study demonstrates that when a two-domain form of the GR lacking LBD but containing the entire NTD region through the complete DBD binds to a DNA oligomer containing a complete palindromic GRE, secondary and tertiary structure is induced in the NTD/AF1 (Kumar et al. 1999). It therefore seems appropriate to think of the DNA sequence of a GRE as an allosteric ligand that can modulate the structural dynamics of the NTD/AF1 (Lefstin and Yamamoto 1998). It has been proposed that this allosteric effect results in a partially folded, "cocked" AF1 conformation, such that it can readily recognize certain coregulatory proteins critical for transcriptional regulation (Kumar and Thompson 2003; Kumar et al. 1999; Kumar and McEwan 2012). This means that a considerable amount of binding energy must be diverted to cause conformational changes in the NTD/AF1 through intramolecular rearrangements. Together, these data suggest that GR and by extension other steroid receptor mechanism of action should include the role of DNA as an allosteric effector, with both local and remote DBD-specific effects. This model of the DNA as allosteric ligand in no way rules out the possibility of further structural modulations in NTD/AF1 (or the entire GR) as a result of steroid binding to LBD, protein:protein interactions involving both AF1 and AF2, and of course post translational modifications.

GR-mediated glucocorticoid signaling is a multifaceted process involving cross talk with various regulatory kinase pathways (Beck et al. 2009; Chen et al. 2008). Thus, signaling cascades that induce phosphorylation of the GR and its coactivator proteins are critical factors in determining the physiological actions of the GR. It has been shown that under physiological conditions, site-specific phosphorylation plays a crucial role in allowing the intrinsically disordered AF1 domain of the GR to adopt functionally active conformation such that the resulting structurally modified forms of AF1 suits for its varied interactions with other critical coregulatory proteins and possibly additional modulations in its structure essential for gene regulation by the GR (Garza et al. 2010; Chen et al. 2008). It has been proposed that these interactions give a set of functionally active folded structure to AF1 region and form the basis for the multiprotein assemblies involved in GR-mediated regulation of transcription (Kumar and Thompson 2012; Kumar and McEwan 2012; Simons et al. 2014). Characterization of phosphorylation and other posttranslational modifications in inducing AF1 structure formation in facilitating protein-protein interactions should be of particular importance in understanding the mechanism by which kinase(s) regulate the transcriptional regulation of the glucocorticoid-mediated target genes in the nucleus. Further, phosphorylation-induced functional conformation in GR AF1 may also shed

light on why most of the major known phosphorylation sites are located in the NTD of the GR and several other nuclear receptors.

There are many examples in which the intrinsically disordered region of a protein undergoes disorder-to-order transition upon interaction with particular binding partner (Reid et al. 2002; McEwan et al. 1996; Kumar and McEwan 2012; Demarest et al. 2002; Kumar et al. 2004a). Coregulatory proteins influence or modulate the transcriptional activity of the GR by multiple mechanisms. In addition to the LXXLL motifs of the p160 steroid receptor coactivators (SRCs) that bind to AF2, an amino-terminal fragment of SRC2/TIF2, binds to the NTD of both PR and GR (Wang et al. 2007) and increases the α-helical content of the GR AF1 domain, suggesting that coactivators augment the transcriptional activity of GR-agonist complexes by inducing more ordered structures beyond the LBD/AF2 region (Khan et al. 2012). Studies have also shown that NTDs of steroid receptors including GR, MR, PR, ER are capable of undergoing a disorder-to-order that results in an increase in α-helical content and tertiary folding upon interaction with the TATA-binding protein (TBP). Additionally, this TBP-induced folding of the NTD was observed to be associated with enhancement of AF1-mediated transcriptional activity (Kumar et al. 2004b; Kumar et al. 2013; Warnmark et al. 2001; Fischer et al. 2010). A potential mechanism by which TBP affects NTD/AF1 activity is to facilitate binding of SRC-1 to NTDs and cooperate with SRC-1 to stimulate NTD-dependent transcriptional activity via reorganizing or stabilizing NTD/AF1 structure for recognition by alternative surfaces of SRC-1 and possibly other proteins in the assembly of coactivator complexes (Kumar et al. 2004b; Khan et al. 2011b; Kumar et al. 2013; Warnmark et al. 2001; Fischer et al. 2010). These data collectively suggest that of the AF1/NTD, rapidly and reversibly adopt multiple structural conformations that can sample the cellular environment until a protein target of proper concentration/affinity are found to selectively stabilize a functional conformation of the NTD. The significance of these findings lies in the possibility of therapeutically targeting AF1 surfaces to achieve tissue-restricted effects with small molecule drugs that can inhibit interactions with protein binding partners such as TBP. Therefore, identifying potential target molecules that could modify AF1-TBP binding may provide the additional selectivity needed to target GR-selective genes and thereby reduce the number of undesirable side effects in current endocrine-based therapies.

4 Influence of Conformational Dynamics in the Regulation of the Cell/Tissue Specific GR Target Genes

The recent X-ray studies of other nuclear receptors such as the structure of the HNF4α homo-dimer bound to DNA and complexed with a coactivator peptide, and the structure of DNA-bound full-length PPARγ/retinoid X receptor (RXR/NR3B1) α heterodimers revealed the possible problems associated with the structure determination (Chandra et al. 2008; Chandra et al. 2013). Further, these studies revealed how allosteric communication between LBD and DBD may trigger extensive physical and functional inter-domain interactions that define their total activity as

opposed to each domain functioning entirely independently. However, these studies failed to account for the structural and functional contributions of the NTD due, mainly, to the role of NTD's flexible conformation that could trigger allosteric regulation within and outside the domain. Allosteric communication between receptor domains has been demonstrated in GR and other nuclear receptors (Chandra et al. 2008; Chandra et al. 2013; Watson et al. 2013; Kumar et al. 1999; Zhang et al. 2011; Helsen et al. 2012; Brodie and McEwan 2005).

Solution phase structural studies using small-angle X-ray scattering, single-particle cryo-electron microscopy and/or hydrogen-deuterium exchange of intact RXR/VDR heterodimers complexed with direct repeat response element DNA have suggested that NTD may have a direct role in modulating DNA binding (Rochel et al. 2011; Osz et al. 2012; Orlov et al. 2012; Dai et al. 2008; Zhang et al. 2011). Further, depending on the precise DNA sequences bound by RXR/VDR heterodimers, distinct domains outside of their DBDs adopt alternative conformations or changed dynamics, indicative of long-range allosteric communication (Rochel et al. 2011; Osz et al. 2012; Orlov et al. 2012; Dai et al. 2008; Zhang et al. 2011). Additional hydrogen-deuterium exchange studies indicated that depending upon the ligand binding, allosteric communication between LBD and DBD can occur in both directions and conformational changes can be propagated between receptor partners (Chandra et al. 2013; Watson et al. 2013; Devarakonda et al. 2011). These complementary approaches to examine nuclear receptor complexes in solution have helped to understand the conformational dynamics and integration of multiple domains of the intact receptor required to regulate different activation states. These observations highlight how several inputs are structurally integrated between distinct functional domains to define the function of multidomain transcriptional regulatory factors including GR and other nuclear receptor proteins.

It has become quite evident from these studies and several failed studies which were never reported in the literature that the main culprit in not getting a crystal structure of GR and other steroid receptors is the intrinsically disordered NTD, which may play the most critical role in the regulation of structural dynamics and the allosteric coupling involving various receptor domains and other coregulators. It thus is axiomatic that attempts to more precisely control GR selectivity with small molecule ligands and cofactors during differential control of gene expression without understanding the functionally active structural features of the NTD will be of limited success. Recent developments provide new insights of how structural flexibility plays an important role in GR's allosteric regulation leading to the fine tuning of target gene expression and the challenges for drug targeting to more precisely control GR activity. Other observations have also led to the conclusion that under cellular conditions, the kinetics of GR when interacting with the DNA and/or other coregulators is highly dynamic and reversible. (McNally et al. 2000; Stenoien et al. 2001; Nagaich et al. 2004).

The GR is known to move to different promoter sites in cells to create a productive assembly of coregulatory proteins for optimal function, and various constellations of such partners are therefore required to associate with the GR as they act specifically to activate or repress glucocorticoid-dependent target genes (McNally et al. 2000; Stenoien et al. 2001; Nagaich et al. 2004; Hittelman et al. 1999). This has particularly been shown in case of agonist vs antagonist bound receptor interacting with differing

sets of coregulatory proteins in a cell/tissue dependent manner. Though some of this behavior has been explained by the effects of specific LBD ligands on the structural dynamics of AF2 and consequent preferential binding of co-activator or co-repressor yet the data so far fall short of fully explaining GR actions most likely due, mainly to, lack of detailed examination of the contributions of NTD/AF1.

Studies have shown that due to allosteric regulations, the flow of information can occur in the opposite direction, as ligand binding induced structural changes in the DBD of VDR (Chandra et al. 2013; Watson et al. 2013). We propose that the integration of various inputs acting on distinct domains of proteins shapes the structure and dynamics of transcriptional regulatory factors, thus allowing them to have context-specific activities. Alternative splicing can generate proteins in which the integration of these signals is altered by rewiring the connections between protein domains, thus allowing different responses to the same signal inputs. This suggests that the GR mediates bidirectional allosteric signaling between the DNA:protein interface and other regulatory domains to specify such context-specific activities in gene regulation (Fig. 5).

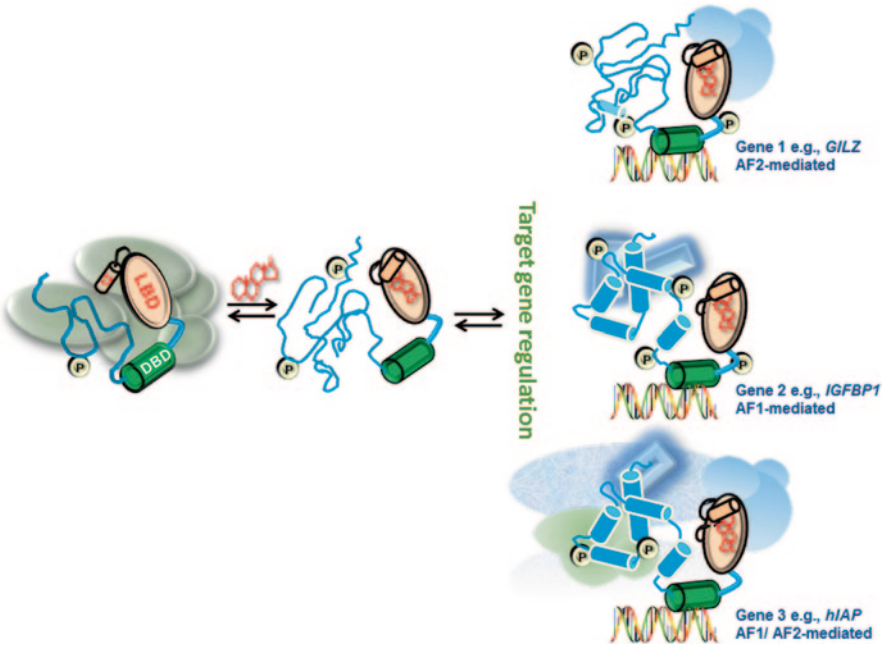


Fig. 5 Allosteric regulator of the conformational dynamics of the GR in transcriptional regulation: Unliganded receptor associated with chaperone proteins. Binding of steroid in the ligand binding pocket and GRE can pass the signal to the surface of the LBD and dynamically reorient AF2 conformation (to attract co-activators) and possibly other parts of the domain. Signals may then be passed to the hinge region, resulting in conformational rearrangements in the hinge, transferring to the DBD and eventually to intrinsically disordered NTD/AF1. This leads to expression of ligand/AF2-mediated target genes. Direct binding of a coregulatory protein to NTD/AF1 and possibly posttranslational modifications can induce structure in the NTD/AF1 and possibly in other part of the receptor. The induced NTD/AF1 conformation suits well for its interaction with co-activators and other receptor domains. This leads to expression of AF1-mediated target genes. Cell/tissue-specific optimal receptor activity requires a synergistic effect of both AF1 and AF2

5 Summary and Perspectives

According to WHO, inflammation related illnesses are one of the biggest challenges in current medicine. As the costs of treating these patients mount up and life comfort and expectancy are threatened, understanding and resolving inflammation is currently one of the main targets in science. Glucocorticoid-based therapy is still the most commonly used treatment to combat chronic and acute inflammation Beck et al. 2009; Hoes et al. 2010. The real challenge is not only to develop more specific ligands, but to change the spectrum of GR-mediated events and try to skew it more towards desired pathways. Therefore, the mainstay of research efforts must be focused on further characterizing the mechanisms of GR actions in detail and developing new therapeutic strategies to fight diseases with a better benefit-to-risk-ratio.

Protein allostery is critical to concepts of combinatorial control and structural studies suggest how signaling information residing in small molecule ligands is transmitted to a coregulatory protein recognition surface. It is also important to note that structural changes at the DNA-binding interface coupled with changes in the GR DBD dimerization interface have been correlated with distinct transcriptional outcomes. These findings provide the mechanistic perspective showing that both DNA and small molecule ligands direct interactions with specific coregulators. In addition, the role of binding partner proteins as ligand to otherwise intrinsically disordered NTD/AF1 in affecting gene-specific regulation could begin to define a molecular map that integrates GREs, ligands, chromatin, coregulators and post-transcriptional modifications to determine the composition and function of gene-specific transcriptional regulatory complexes involving GR.

The most widely used small molecule GR modulators have been designed to bind the structured ligand binding pocket. Clinically this phenomenon has extensively been exploited; however, it has been suggested that the cell/tissue-specific residual activity of GR in the presence of a ligand may, mainly, be mediated via AF1. Thus, to develop novel strategies to therapeutically target AF1 should provide additional selectivity needed for tissue/gene-specific GR targets in current endocrine-related therapies. Therefore, blocking AF1-target binding protein interaction sites may provide potential avenues for additional selectivity to target cell-tissue specific gene regulations of GR that could complement or replace existing small molecule steroidal ligand actions in current endocrine-based therapies. We therefore propose that mainstream research must focus on: (1) Defining the structural and functional consequences of AF1-target binding protein interactions; (2) Determineing the effects of target binding protein-induced folding on AF1's interactions with GR-coactivator proteins and AF1 activity; and 3) Developing inhibitors of AF1-target binding protein interaction as novel GR therapeutic target.

References

Ahmad N, Kumar R (2011) Steroid hormone receptors in cancer development: a target for cancer therapeutics. Cancer Lett 300:1–9

Almlof T, Wallberg AE, Gustafsson JA, Wright AP (1998) Role of important hydrophobic amino acids in the interaction between the glucocorticoid receptor tau1-core activation domain and target factors. Biochemistry 37:9586–9594

Barnes PJ (1998) Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Lond) 94:557–72

Baskakov IV, Kumar R, Srinivasan G, Ji Y, Bolen DW, Thompson EB (1999) Trimethylamine N-oxide-induced cooperative folding of an intrinsically unfolded transcription-activating fragment of human glucocorticoid receptor. J Biol Chem 274:10693–10696

Baumann H, Paulsen K, Kovacs H, Berglund H, Wright AP, Gustafsson JA, Hard T (1993) Refined solution structure of the glucocorticoid receptor DNA binding domain. Biochemistry 32:13463–13471

Beato M, Chavez S, Truss M (1996) Transcriptional regulation by steroid hormones. Steroids 61:240–251

Beck IM, Vanden Berghe W, Vermeulen L, Yamamoto KR, Haegeman G, De Bosscher K (2009) Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. Endocr Rev 30:830–882

Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE (2002) Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell 110:93–105

Brodie J, McEwan IJ (2005) Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. J Mol Endocrinol 34:603–615

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758

Carson MW, Luz JG, Suen C, Montrose C, Zink R, Ruan X, Cheng C, Cole H, Adrian MD, Kohlman DT, Mabry T, Snyder N, Condon B, Maletic M, Clawson D, Pustilnik A, Coghlan MJ (2014) Glucocorticoid receptor modulators informed by crystallography lead to a new rationale for receptor selectivity, function, and implications for structure-based design. J Med Chem 57:849–860

Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, Burris TP, Rastinejad F (2008) Structure of the intact PPAR-gamma-RXR-nuclear receptor complex on DNA. Nature 456:350–356

Chandra V, Huang P, Potluri N, Wu D, Kim Y, Rastinejad F (2013) Multidomain integration in the structure of the HNF-4α nuclear receptor complex. Nature 495:394–398

Chen W, Dang T, Blind RD, Wang Z, Cavasotto CN, Hittelman AB, Rogatsky I, Logan SK, Garabedian MJ (2008) Glucocorticoid receptor phosphorylation differentially affects target gene expression. Mol Endocrinol 22:1754–1766

Dahlman-Wright K, Almlöf T, McEwan IJ, Gustafsson JA, Wright AP (1994) Delineation of a small region within the major transactivation domain of the human glucocorticoid receptor that mediates transactivation of gene expression. Proc Natl Acad Sci USA 91:1619–1623

Dahlman-Wright K, Baumann H, McEwan IJ, Almlöf T, Wright AP, Gustafsson JA, Härd T (1995) Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor. Proc Natl Acad Sci USA 92:1699–1703

Dai SY, Chalmers MJ, Bruning J, Bramlett KS, Osborne HE, Montrose-Rafizadeh C, Barr RJ, Wang Y, Wang M, Burris TP, Dodge JA, Griffin PR (2008) Prediction of the tissue specificity of selective estrogen receptor modulators by using a single biochemical method. Proc Natl Acad Sci USA 105:7171–7176

De Bosscher K, Vanden Berghe W, Haegeman G (2003) The interplay between the glucocorticoid receptor and nuclear factor kappa B or activator protein-1: molecular mechanisms for gene repression. Endocr Rev 24:488–522

De Bosscher K, Haegeman G, Elewaut D (2010) Targeting inflammation using selective glucocorticoid receptor modulators. Curr Opin Pharmacol 10:497–504

Demarest SJ, Martinez-Yamout M, Chung J, Chen H, Xu W, Dyson HJ, Evans RM, Wright PE (2002) Mutual synergistic folding in recruitment of CBP/p300 by p160 nuclear receptor coactivators. Nature 415:549–553

Devarakonda S, Gupta K, Chalmers MJ, Hunt JF, Griffin PR Van Duyne GD, Spiegelman BM (2011) Disorder-to-order transition underlies the structural basis for the assembly of a transcriptionally active PGC-1α/ERRa complex. Proc Natl Acad Sci USA 108:18678–18683

Eriksson MA, Nilsson L (1995) Structure, thermodynamics and cooperativity of the glucocorticoid receptor DNA-binding domain in complex with different response elements. Molecular dynamics simulation and free energy perturbation studies. J Mol Biol 253:453–572

Evans RM (1995) The steroid and thyroid hormone receptor superfamily. Science 240:889–895

Fischer K, Kelly SM, Watt K, Price NC, McEwan IJ (2010) Conformation of the mineralocorticoid receptor N-terminal domain: evidence for induced and stable structure. Mol Endocrinol 24:1935–1948

Ford J, McEwan IJ, Wright AP, Gustafsson JA (1997) Involvement of the transcription factor IID protein complex in gene activation by the N-terminal transactivation domain of the glucocorticoid receptor in vitro. Mol Endocrinol 11:1467–1475

Frankfurt O, Rosen ST (2004) Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. Curr Opin Oncol 16:553–563

Frey FJ, Odermatt A, Frey BM (2004) Glucocorticoid-mediated mineralocorticoid receptor activation and hypertension. Curr Opin Nephrol Hypertens 13:451–458

Garza AS, Khan SH, Kumar R (2010) Site-specific phosphorylation induces functionally active conformation in the intrinsically disordered N-terminal activation function domain (AF1) of the glucocorticoid receptor. Mol Cell Biol 30:220–230

Härd T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson JA, Kaptein R (1990) Solution structure of the glucocorticoid receptor DNA-binding domain. Science 249:157–160

He Y, Yi W, Suino-Powell K, Zhou XD, Tolbert WD, Tang X, Yang Z, Yang H, Shi J, Hou L, Jiang H, Melcher K, Xu HE (2014) Structures and mechanism for the design of highly potent glucocorticoids. Cell Res 24:713–726

Helsen C, Dubois V, Verfaillie A, Young J, Trekels M, Vancraenenbroeck R, De Maeyer M, Claessens F (2012) Evidence for DNA-binding domain—ligand-binding domain communications in the androgen receptor. Mol Cell Biol 32:3033–3043

Henriksson J, Almlof T, Ford J, McEvan IJ, Gustafsson JA, Wright AP (1997) Role of Ada adapter complex in gene activation by the glucocorticoid receptor. Mol Cell Biol 17:3065–3073

Hittelman AB, Burakov D, Iniguez-Lluhi JA, Freedman LP, Garabedian MJ (1999) Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. EMBO J 18:5380–5388

Hoes JN, Jacobs JW, Buttgereit F, Bijlsma JW (2010) Current view of glucocorticoid co-therapy with DMARDs in rheumatoid arthritis. Nat Rev Rheumatol 6:693–702

Hollenberg SM, Giguère V, Segui P, Evans RM (1987) Colocalization of DNA binding and transcriptional activation functions in the human glucocorticoid receptor. Cell 49:39–46

Inaba H, Pui CH (2010) Glucocorticoid use in acute lymphoblastic leukaemia. Lancet Oncol 11:1096–1106

Khan SH, Arnott JA, Kumar R (2011a) Naturally occurring osmolyte, trehalose induces functional conformation in an intrinsically disordered region of the glucocorticoid receptor. PLoS ONE 6:e19689

Khan SH, Ling J, Kumar R (2011b) TBP binding-induced folding of the glucocorticoid receptor AF1 domain facilitates its interaction with steroid receptor coactivator-1. PLoS ONE 6:e21939

Khan SH, Awasthi S, Guo C, Goswami D, Ling J, Griffin PR, Simons SS, Kumar R (2012) Binding of the amino terminal region of coactivator TIF2 to the intrinsically disordered AF1 domain of the glucocorticoid receptor is accompanied by conformational reorganizations. J Biol Chem 287:44546–44560

Kumar R, McEwan IJ (2012) Allosteric modulators of steroid hormone receptors: structural dynamics and gene regulation. Endocri Re 33:271–299

Kumar R, Thompson EB (1999) The structure of the nuclear hormone receptors. Steroids 64: 310–319

Kumar R, Thompson EB (2003) Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. Mol Endocrinol 17:1–10

Kumar R, Thompson EB (2005) Gene regulation by the glucocorticoid receptor: structure:function relationship. J Steroid Biochem Mol Biol 94:383–394

Kumar R, Thompson EB (2010) Intramolecular signaling between the glucocorticoid receptor AF1 and DNA binding domains: Influence of AF1 flanking sequences within the N-terminal domain on the structure and functions of AF1. Arch Biochem Biophys 496:140–145

Kumar R, Thompson EB (2012) Folding of the glucocorticoid receptor N-terminal activation domain: dynamics and regulation. Mol Cell Endo 348:450–456

Kumar R, Baskakov IV, Srinivasan G, Bolen DW, Lee JC, Thompson EB (1999) Inter-domain signaling in a two-domain fragment of the human glucocorticoid receptor. J Biol Chem 274:24737–24741

Kumar R, Lee JC, Bolen DW, Thompson EB (2001) Osmolyte induced conformation of the glucocorticoid receptor AF1/tau1 domain binds coregulators. J Biol Chem 276:18146–18152

Kumar R, Betney R, Li J, Thompson EB, McEwan IJ (2004a) Induced alpha-helix structure in AF1 of the androgen receptor upon binding transcription factor TFIIF. Biochemistry 43:3008–3013

Kumar R, Volk DE, Li J, Lee JC, Gorenstein DG, Thompson EB (2004b) TATA box binding protein induces structure in the recombinant glucocorticoid receptor AF1 domain. Proc Natl Acad Sci USA 101:16425–16430

Kumar R, Serrette JM, Khan SH, Miller AL, Thompson EB (2007) Differential effects of osmolytes on the induced folding of the glucocorticoid receptor AF1 domain. Arch Biochem. Biophys 465:452–460

Kumar R, Moure CM, Khan SH, Callaway C, Grimm S, Goswami D, Griffin PR, Edwards DP (2013) Regulation of the structurally dynamic disordered amino-terminal domain of progesterone receptor by protein induced folding. J Biol Chem 288:30285–30299

Lefstin JA, Yamamoto KR (1998) Allosteric effects of DNA on transcriptional regulators. Nature 392:885–888

Lu NZ, Cidlowski JA (2005) Translational regulatory mechanisms generate N terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell 18:331–342

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505

McEwan IJ, Dahlman-Wright K, Ford J, Wright AP (1996) Functional interaction of the c-Myc transactivation domain with TATA binding protein: evidence for an induced fit model of transactivation domain folding. Biochemistry 35:9584–9593

McNally JG, Muller WG, Walker D, Wolford R, Hager GL (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. Science 287:1262–1265

Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324:407–410

Miesfeld R, Godowski PJ, Maler BA, Yamamoto KR (1987) Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. Science 236:423–427

Nagaich AK, Walker DA, Wolford R, Hager GL (2004) Rapid periodic binding and displacement of the glucocorticoid receptor during chromatin remodeling. Mol Cell 14:163–174

Oakley RH, Cidlowski JA (2011) Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. J Biol Chem 286:3177–3184

Oakley RH, Cidlowski JA (2013) The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J Allergy Clin Immunol 132:1033–1044

Orlov I, Rochel N, Moras D, Klaholz BP (2012) Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. EMBO J 31:291–300

Osz J, Pethoukhov MV, Sirigu S, Svergun DI, Moras D, Rochel N (2012) Solution structures of PPAR/RXR complexes. PPAR Res 701412:2012

Pratt WB, Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18:306–360

Prilusky J, Felder CE, Zeev-Ben-Mordehai T, Rydberg EH, Man O, Beckman JS, Silman I, Sussman JL (2005) FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics 21:3435–3438

Reichardt HM, Schutz G (1998) Glucocorticoid signalling–multiple variations of a common theme. Mol Cell Endocrinol 146:1–6

Reid J, Kelly SM, Watt K, Price NC, McEwan IJ (2002) Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. J Biol Chem 277:20079–20086

Rochel N, Ciesielski F Godet J, Moman E, Roessle M, Peluso-Iltis C, Moulin M, Haertlein M, Callow P, Mély Y, Svergun DI, Moras D (2011) Common architecture of nuclear receptor heterodimers on DNA direct repeat elements with different spacings. Nat Struct Mol Biol 18:564–570

Rosen J, Miner JN (2005) The search for safer glucocorticoid receptor ligands. Endocr Rev 26:452–464

Schiller BJ, Chodankar R, Watson LC, Stallcup MR, Yamamoto KR (2014) Glucocorticoid receptor binds half sites as a monomer and regulates specific target genes. Genome Biol 15:418

Schoch GA, D'Arcy B, Stihle M, Burger D, Bär D, Benz J, Thoma R, Ruf A (2010) Molecular switch in the glucocorticoid receptor: active and passive antagonist conformations. J Mol Biol 395:568–577

Simons SS Jr (2008) What goes on behind closed doors: physiologicalversus pharmacological steroid hormone actions. Bioessays 30:744–756

Simons SS Jr (2010) Glucocorticoid receptor cofactors as therapeutic targets. Curr Opin Pharmacol 10:613–619

Simons SS, Edwards DP, Kumar R (2014) Dynamic Structures of Nuclear Hormone Receptors: New Promises and Challenges. Mol Endocrinol 28:173–82

Stenoien DL, Patel K, Mancini MG, Dutertre M, Smith CL, O'Malley BW, Mancini MA (2001) FRAP reveals that mobility of oestrogen receptor is ligand- and proteasome dependent. Nat Cell Biol 3:15–23

Tao YG, Xu Y, Xu HE, Simons SS Jr (2008) Mutations of glucocorticoid receptor differentially affect AF2 domain activity in a steroid-selective manner to alter the potency and efficacy of gene induction and repression. Biochemistry 47:7648–7662

Thompson EB, Johnson BH (2003) Regulation of a distinctive set of genes in glucocorticoid-evoked apoptosis in CEM human lymphoid cells. Recent Prog Horm Res 58:175–197

Wang D, Wang Q, Awasthi S, Simons SS Jr (2007) Amino-terminal domain of TIF2 is involved in competing for corepressor binding to glucocorticoid and progesterone receptors. Biochemistry 46:8036–8049

Warnmark A, Gustafsson JA, Wright AP (2000) Architectural principles for the structure and function of the glucocorticoid receptor tau1 core activation domain. J Biol Chem 275:15014–15018

Warnmark A, Wikstrom A, Wright AP, Gustafsson JA, Hard T (2001) The N-terminal regions of estrogen receptor a and b are unstructured in vitro and show different TBP binding properties. J Biol Chem 276:45939–45944

Watson LC, Kuchenbecker KM, Schiller BJ, Gross JD, Pufall MA, Yamamoto KR (2013) The glucocorticoid receptor dimer interface allosterically transmits sequence-specific DNA signals. Nat Struct Mol Biol 20:876–883

Yudt MR, Cidlowski JA (2001) Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Mol Endocrinol 15:1093–1103

Zhang J, Chalmers MJ, Stayrook KR, Burris LL, Wang Y, Busby SA, Pascal BD, Garcia-Ordonez RD, Bruning JB, Istrate MA, Kojetin DJ, Dodge JA, Burris TP, Griffin PR (2011) DNA binding alters coactivator interaction surfaces of the intact VDR-RXR complex. Nat Struct Mol Biol 18:556–563

What Determines the Difference in DNA Binding Between the Androgen and the Glucocorticoid Receptors?

Vanessa Dubois, Christine Helsen, Liesbeth Clinckemalie, Lien Spans, Michaël Laurent, Leen Antonio, Thomas Van den Broeck, Stefan Prekovic, Steven Joniau, Dirk Vanderschueren and Frank Claessens

1 Introduction

Androgens play crucial physiological roles in establishing and maintaining the male phenotype. Their actions are essential for the differentiation and growth of the male reproductive organs, initiation and regulation of spermatogenesis, and the control of male sexual behavior. In addition, androgens also have anabolic actions on several extragenital structures including muscle and bone (Dubois et al. 2012; Laurent et al. 2013). Glucocorticoids are crucial regulators of glucose metabolism and inflammatory processes (So et al. 2007). To exert this variety of biological processes, the hormones bind their cognate receptors being the androgen and glucocorticoid receptors (AR/NR3C4 and GR/NR3C1) that act as ligand-inducible transcription factors to control the expression of target genes (Claessens et al. 2008).

AR and GR are members of the steroid receptor family—a subfamily of nuclear receptors—to which the progesterone, mineralocorticoid and estrogen receptors (PR/NR3C3, MR/NR3C2, ERα/NR3A1 and ERβ/NR3A2) also belong. Steroid

F. Claessens (✉)
Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven, Campus Gasthuisberg O&N1 Herestraat 49, PO Box 901, 3000 Leuven, Belgium
e-mail: frank.claessens@med.kuleuven.be

V. Dubois · C. Helsen · L. Clinckemalie · L. Spans · M. Laurent · L. Antonio · T. Van den Broeck · S. Prekovic
Molecular Endocrinology Laboratory, KU Leuven, Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

M. Laurent
Division of Gerontology and Geriatrics, KU Leuven, Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

L. Antonio · D. Vanderschueren
Division of Clinical and Experimental Endocrinology, KU Leuven, Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

T. Van den Broeck · S. Joniau
Division of Urology, KU Leuven, Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_4

receptors are built in a modular fashion and consist of an amino-terminal domain (NTD), a central DNA-binding domain (DBD), a hinge region, and a carboxy-terminal ligand-binding domain (LBD). Once activated by their cognate ligand, they bind with high specificity to DNA response elements found in the regulatory regions of target genes and recruit several cofactors, ultimately leading to gene transcription (Tsai et al. 1994).

The AR depends on a strong amino/carboxy-terminal (N/C)-interaction for its full activity (Ikonen et al. 1997). This N/C-interaction involves an FQNLF core sequence within the NTD that has the ability to bind an activation function in the LBD in an androgen-dependent manner (Alen et al. 1999; Ma et al. 1999). Other features of the AR-NTD influencing the receptor transcriptional activity include a polyglutamine stretch of variable length (Callewaert et al. 2003a; Irvine et al. 2000), and the sumoylation status of two lysine residues (Callewaert et al. 2004; Kotaja et al. 2002). For the GR, both the NTD and the LBD are strong transcription activation domains interacting with components of Mediator and the p160 coactivators, respectively (McEwan et al. 2007).

The DBDs of steroid receptors consist of two so-called zinc fingers, which are zinc-nucleated modules in which four cysteine residues are coordinated by a zinc atom. The first zinc finger makes sequence-specific contacts with the DNA, while the second is involved in receptor dimerization (Claessens et al. 2008). However, a receptor fragment that only covers the two zinc fingers has no or very low affinity for DNA. For the AR, it was shown that the minimal DBD has to include a carboxy-terminal extension (CTE) for high affinity DNA binding (Schoenmakers et al. 1999; Tanner et al. 2010).

Despite the very different physiological effects of steroids, there are strong similarities in the DNA sites recognized by their respective receptors (Cato et al. 1987), and individual response elements from target genes of different hormones can even be identical in sequence (Claessens et al. 2001). Moreover, the DBDs of the GR, PR, MR, and AR resemble each other more than they resemble the DBDs of the other nuclear receptors (Claessens et al. 2004). This promiscuity raises the question of how selectivity of hormone action is achieved. Several hypotheses have been proposed to explain this selectivity, and these hypotheses are not mutually exclusive. Obvious mechanisms include differential expression of receptors or coregulators, and tissue-specific metabolism of activating ligands. Tissue-specific expression of pioneer factors with receptor-specific actions may also account for a part of the selectivity, as illustrated by the key role of FoxA1 in ER function (Hurtado et al. 2011). Finally, differences in chromatin structure and organization can also play an important role in the selectivity of hormone action (Verrijdt et al. 2003). In this chapter, we will focus on the DNA motifs recognized by the AR and GR, and how selectivity is achieved through subtle differences in the DNA elements that receptor DBDs recognize on chromatin. A clear difference between AR and GR is the ability to be recruited to DNA via other transcription factors. While this has been documented well for the GR, it is far less clear how important this mechanism is for the AR. The in vivo role of the different types of motifs will be highlighted, as well as the complexity of the enhancers containing these motifs. Finally, we will discuss how androgen response elements, besides recruiting the AR to regulatory

regions of androgen target genes, also impart information to the receptor by acting as allosteric modulators.

2 Androgen Response Elements

Like most transcription factors, the AR and GR have to find specific DNA motifs in enhancers which are scattered all over the genome. While the inactive part of the genome is packed into heterochromatin and thus invisible to most transcription factors, the response elements in regulatory regions of the target genes can be in more open chromatin. It is still unclear how exactly the AR can find them, although growing evidence points at several pioneering factors like FoxA1 and GATA2 that either recruit the AR to its cognate DNA motifs or are prebound to the enhancers making the enhancer available through the eviction of the nucleosome (Lupien et al. 2009; Tewari et al. 2012; Wang et al. 2007). In this section, however, we will restrict ourselves to the description of DNA elements that are recognized by the DBDs, the so-called androgen and glucocorticoid response elements.

An androgen or glucocorticoid response element (ARE, GRE) is defined as a simple DNA motif, able to convey androgen/glucocorticoid-responsiveness to a heterologous reporter gene through direct binding of the AR/GR. Experimentally defining an ARE/GRE involves in vitro binding experiments like electrophoretic mobility shift assay (EMSA) or DNase I footprinting on the one hand, and transfection assays on the other (De Bruyn et al. 2011). Ultimate proof for a response element is the demonstration of receptor binding in a chromatin immunoprecipitation assay (ChIP) in a cellular context, and receptor activity in a transgenic model in which the element is mutated. However, the latter approach needs to be considered with care. Indeed, deleting one response element is most likely insufficient to abolish gene regulation, since an individual gene can be controlled by several enhancers as discussed in more detail in a later section.

2.1 Classical AREs

Historically, the consensus high affinity binding sequence for the GR was described to be 5′-AGAACA-3′ (Payvar et al. 1983). It became clear that the AR also recognizes this motif in the glucocorticoid responsive MMTV enhancer (Parker et al. 1987). Further research showed that, three nucleotides 3′ of this high affinity binding site, a second binding site is present with a similar consensus but in the other strand (Claessens et al. 1989; Roche et al. 1992). Indeed, the AR binds DNA as a homodimer, binding two 5′-AGAACA-3′-like motifs separated by a three nucleotide spacer and organized as an inverted repeat (Fig. 1). These DNA motifs, hereafter referred to as classical AREs, are not only recognized by AR and GR, but also display affinity for PR and MR (Denayer et al. 2010).

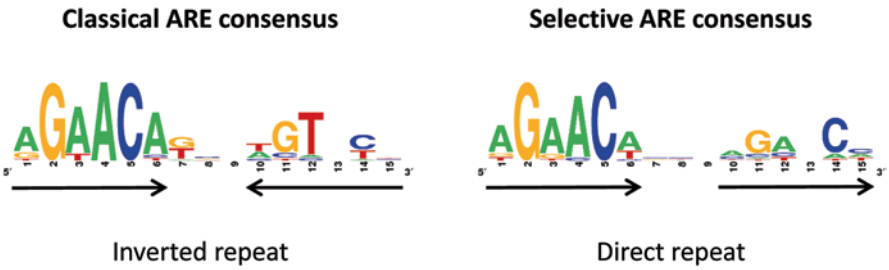


Fig. 1 Sequence logos for classical and selective AREs. Classical AREs resemble inverted repeats of the 5′-AGAACA-3′-like motif spaced by three nucleotides, while selective AREs resemble direct repeats of the same motif. Logos were created on the weblogo.berkeley.edu website, based on a list of all published AREs for which selectivity was checked in electrophorectic mobility shift assays and in functional analysis

2.2 Selective AREs

The DBD of AR, GR, PR, and MR are very similar, with identity of the residues involved in contacting the DNA and high similarity of the dimerization interface. However, since each corresponding hormone has its specific target genes, even in cells where the receptors are co-expressed, efforts have been made to find DNA sequences that are selective for any of the four receptors. In vitro DNA motif selections, based on PCR amplifications of DBD-bound oligonucleotides, did not reveal selective elements (Roche et al. 1992). It was only through the analysis of a series of AREs isolated from androgen target genes that it became apparent that several of these AREs were not recognized by the GR-DBD. These so-called selective AREs consist of a 5′-AGAACA-3′-like hexamer, flanked at three nucleotides downstream by a second hexamer. The similarity of this downstream hexamer to the 5′-AGAACA-3′ consensus is lower compared to that in the classical AREs. In fact, initial mutation analyses indicated that selective AREs resemble a direct, rather than a palindromic, repeat of the 5′-AGAACA-3′ consensus (Claessens et al. 1996; Verrijdt et al. 2000) (Fig. 1).

For the DBD of the ER, residues in the first zinc finger module dictate higher affinity for 5′-AGGTCA-3′, while in GR, AR, PR and MR, alternative residues at the same positions dictate high affinity for 5′-AGAACA-3′ (Umesono et al. 1989). Since the two hexamers that constitute all AREs, both classical and selective, resemble the same consensus, it is not surprising that the binding of the AR to selective AREs and the non-binding of the GR to these elements is not determined by differences in the first zinc finger. However, a chimeric AR in which the second zinc finger of the DBD is replaced by that of the GR possesses significantly attenuated affinity for selective AREs in transient transfections experiments and in vitro binding assays (Schoenmakers et al. 1999). This finding indicates that the second zinc finger of the AR allows dimerization on selective elements, while the second zinc finger of the GR does not.

2.2.1 In vivo Role

Based on the in vitro data on the role of the second zinc finger in selective ARE binding, and the fact that this receptor fragment is encoded by a separate exon in the AR as well as in the GR gene, a transgenic model was developed in which this exon in the AR gene was swapped by that of the GR gene. The resulting model, called SPARKI for 'SPecificity affecting AR Knock In' expresses an AR that still binds classical AREs with high affinity but has lost high affinity for selective AREs (Schauwaers et al. 2007). In effect, this model can be considered a knockout of selective AREs. These mice have smaller reproductive organs and reduced fertility, while no differences are observed in other androgen target tissues like bone, muscle, kidney or lacrimal glands. Therefore, it seems that selective AREs are not involved in the anabolic effects of androgens, but have a specific role in reproduction.

The reduced fertility observed in SPARKI is mainly explained at two sites: in the testis, the number of Sertoli cells is reduced and the spermatogenic process seems to be affected at the second meiotic division; in the epididymis, the sperm maturation is impaired and this correlates with the reduced expression of a subset of the androgen-regulated genes in this tissue. Several of these genes have a known role in sperm maturation, and selective AREs were identified in two of them which is proof for the fact that the second zinc finger of the AR has a crucial role in the recognition of selective AREs (Kerkhofs et al. 2012).

Several of the AREs described in AR-binding segments found in human prostate cancer cell lines are selective AREs (Denayer et al. 2010; Wang et al. 2007). The fact that the prostate of SPARKI mice is smaller (Schauwaers et al. 2007) indicates that selective AREs have a role in the development of normal prostates, but it still is unclear whether this type of AREs is also involved in the etiology or evolution of prostate cancer.

2.2.2 Mechanism of Selectivity

As discussed above, the second zinc finger plays an essential role in the binding of AR and the non-binding of GR to selective AREs. This suggests that the discrimination between the direct repeat motifs and the classical palindromes is not determined by a difference in monomeric sequence recognition, but rather by a difference in dimerization of the receptor DBDs (Helsen et al. 2012b; Verrijdt et al. 2006). Compared to that of the GR, the AR second zinc finger differs at four positions. Two of them have been demonstrated to be involved in the stronger dimerization of AR compared to GR by creating additional interactions that stabilize the AR dimer interface (Shaffer et al. 2004), but in functional assays, swapping these residues between AR and GR DBDs had no effect on their selectivity (Verrijdt et al. 2006). However, removal of the carboxy-terminal part of the CTE containing these two residues abolishes AR binding to selective AREs but has no effect on AR-DBD interaction with classical AREs (Schoenmakers et al. 2000). Deletions and point mutations in the full size receptor have similar effects on DNA binding (Haelens

et al. 2007; Tanner et al. 2010). Thus, the stronger dimerization function in the AR-DBD seems to allow the AR to dimerize on response elements that are divergent from the ARE consensus, e.g. organized as direct repeats instead of the canonical inverted repeats.

A recent study addressed the role of the DBD in ensuring specificity of AR binding to chromatin in vivo. In that study, AR binding events were compared between wild type and SPARKI mice (Sahu et al. 2014). As discussed above, SPARKI mice express an AR that still binds classical AREs but has lost high affinity for selective AREs. Motif search analysis indicated that, as expected, an inverted palindromic repeat resembling the ARE consensus is highly enriched among the AR binding sites preferred by SPARKI AR, with a highly conserved threonine at position 12 (Sahu et al. 2014). On the other hand, analysis of the binding sites preferred by wild type AR uncovered a different element in which the 5' hexamer is almost identical to the 5′-AGAACA-3′ consensus but followed by a second hexamer with weak sequence conservation (Sahu et al. 2014). Interestingly, the very highly conserved thymidine at position 12 present at the SPARKI AR-preferred sites is dispensable among the elements for wild type AR binding (Sahu et al. 2014). These data indicate that AR selectivity is achieved by relaxed rather than increased element stringency at chromatin binding sites which, in turn, attenuates binding of other steroid receptors to these elements. In line with this, genome-wide ChIP-seq experiments on several cell lines and tissues have shown that response elements for GR contain a highly conserved thymidine at position 12 (Grontved et al. 2013; John et al. 2011). Altogether, these findings support the abovementioned model that the stronger dimerization of AR-DBD allows binding of a second AR molecule on 3′ hexamers that show less resemblance with the 5′-AGAACA-3′ consensus, in contrast to the GR. Interestingly, the PR also seems to have more relaxed DNA binding selectivity compared to GR (Denayer et al. 2010).

2.3 Negative AREs

While many insights have been obtained into how the AR mediates gene activation, the mechanisms underlying AR-mediated gene repression are less understood. Possible actions include downregulation of transcription activators, competition with cofactors, and inhibition of nuclear localization of transcription factors (Grosse et al. 2012). Another way of AR-mediated gene repression may occur through the direct binding of the AR onto the DNA and subsequent formation of corepressor complexes leading to a more condensed, thus repressive, chromatin conformation. These putative AR binding sites in the regulatory regions of androgen-repressed genes were called negative AREs.

Several examples of negative AREs have been described (Lanzino et al. 2010; Qi et al. 2012; Zhang et al. 1997). In these papers, 5′-promotor-deletion mutants were used in reporter gene assays to identify negative AREs within the proximal region of the promotor of the androgen-repressed gene of interest. Furthermore, EMSA experiments indicated a direct interaction of the AR with the suggested ARE in vitro.

Concerning the consensus sequence of these negative AREs, some authors conclude that they exhibit characteristics of partial direct repeats, thus more resembling selective AREs (Lanzino et al. 2010). Others only see similarity with the 5′-AGAACA-3′ consensus in the 5′ part of the negative ARE, whereas the 3′ part does not resemble the hexameric consensus sequence.

While there is no doubt about the repressive effect of androgens on the expression of certain genes, incertitude remains concerning the exact nature of this transcriptional repression. Indeed, while AR binding and transrepression has been showed extensively in vitro, recruitment of androgen-activated AR to these negative AREs in a native chromatin context, e.g. by ChIP analysis, has not been demonstrated yet. Moreover, other transcription factors may participate in negative ARE function by interacting with the 3' half of the response element. This hypothesis is supported by the presence of binding sites for Sp1 (Lanzino et al. 2010) and Ets (Zhang et al. 1997) transcription factors downstream of the described negative AREs. Thus, although androgen-mediated gene repression may involve direct AR binding to specific DNA elements, the nature of these motifs and the mechanisms by which they repress transcription remain elusive.

2.4 AREs are Always Part of Complex Enhancers

Traditionally, experimental searches for AREs have focused on relative short genomic segments just up- and downstream of the transcription start site (Claessens et al. 1989; Cleutjens et al. 1996). In search of a more comprehensive view of androgen signaling, ChIP techniques coupled with genome-wide DNA microarray hybridization (ChIP-on-chip) or massive parallel sequencing (ChIP-seq) have identified the patterns of AR binding in various cell line models (Jia et al. 2008; Takayama et al. 2007; Wang et al. 2007). Rather than identifying a list of target genes whose proximal regulatory elements are occupied by the AR, these studies uncovered a complex mechanism typified by AR binding to distal enhancer elements (>10 kb). Subsequent elucidation of the mechanism by which the AR regulates transcription from afar revealed a close spatial localization of AR-bound enhancers with the promotor regions of androgen-responsive genes, which was called chromatin looping (Taslim et al. 2012; Wang et al. 2005). Hence, AREs are not necessarily in close vicinity of the gene they regulate.

A study that combined RNA expression profiling to identify androgen-responsive genes with ChIP-on-chip to define AR binding regions (Bolton et al. 2007) demonstrated that most androgen-responsive genes are associated with two or more AREs. Moreover, the androgen-responsive genes were sometimes themselves linked in gene clusters, consisting of several androgen target genes and multiple AREs (Bolton et al. 2007). These data suggest combinatorial regulation of individual androgen-responsive genes, adding complexity to the understanding of the androgen regulation of a particular gene. That same study also confirmed the older notion that AREs appear to be composite elements, containing AR binding sequences adjacent to binding motifs for other transcriptional factors (Bolton et al. 2007;

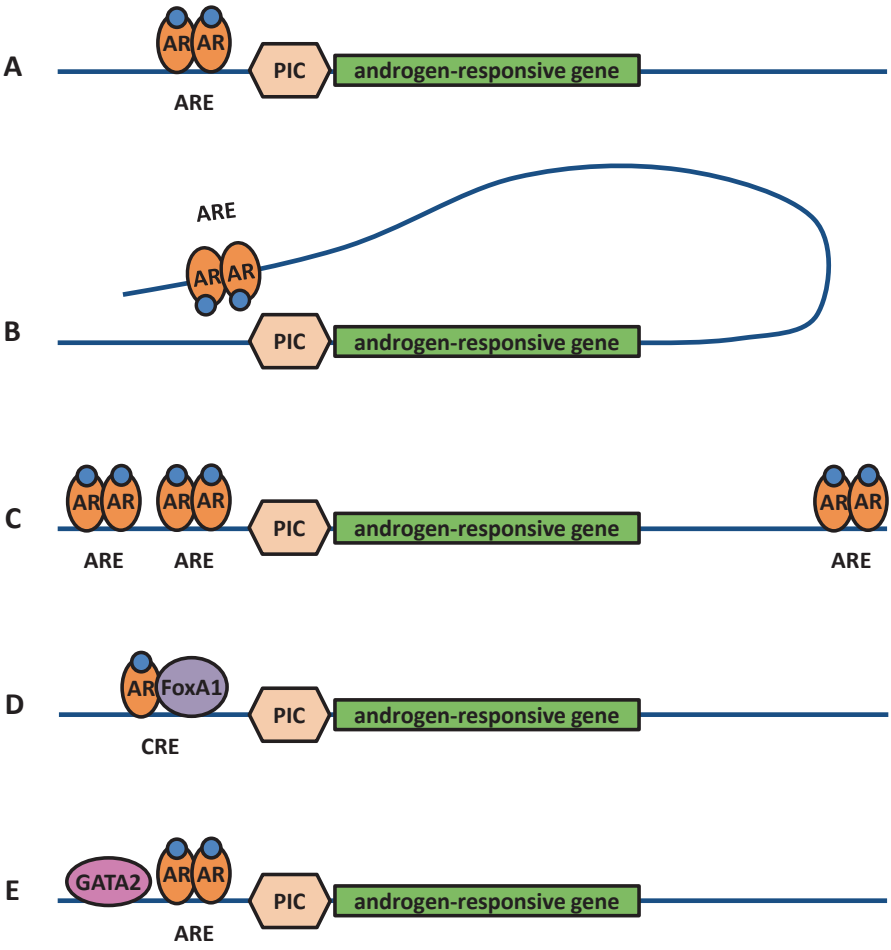


Fig. 2 Androgen response elements are part of complex enhancers. Androgen regulation of a target gene is more complex than androgen receptor (*AR*) binding on a single androgen response element (*ARE*) in the promotor of that gene **a**. Indeed, AREs can also be found in distal regions and make contact with the transcription preinitiation complex (*PIC*) by chromatin looping **b**, several AREs can cooperate to regulate an individual gene **c**, and the *AR* may also bind as a heterodimer on a composite response element (*CRE*) together with another transcription factor such as FoxA1 **d**. Additionally, the *AR* may also bind as a homodimer on its *ARE* while a flanking transcription factor such as GATA2 functions as a pioneer factor for AR binding **e**

Claessens et al. 2001). This finding was strengthened by recent AR ChIP-seq data showing that, in some cases, the AR seems to bind DNA elements as a heterodimer with FoxA1 (Sahu et al. 2011).

Thus, androgen regulation of a target gene is more complex than AR binding on a single ARE in the promotor of that gene (Fig. 2). Indeed, AREs can also be found in distal regions, several AREs can cooperate to regulate an individual gene, and the AR may also bind as a heterodimer on a composite binding site together with

another transcription factor. Additionally, as several studies report the presence of binding sites for other transcription factors in close vicinity of AREs, the AR may also bind as a homodimer on its ARE while the flanking transcription factor, e.g. GATA2, functions as a pioneer factor for AR binding (Clinckemalie et al. 2013; Wang et al. 2007).

3 Androgen Response Elements as Allosteric Modulators

While the cognate ligands of the AR are testosterone and dihydrotestosterone, the DNA response elements can also be considered ligands, rather than merely AR docking sites near the androgen target genes. Indeed, there are several lines of evidence that indicate that the response elements can modulate the activity of the bound AR, thereby acting acting as allosteric modulators.

3.1 DNA Binding Imparts Conformational Changes

Following DNA recognition, conformational changes take place in the second zinc finger that favor receptor dimerization (Freedman 1992). As discussed above, multiple AREs are often found in the regulatory regions of androgen-responsive genes. The study of their respective role has led to the concept of cooperativity due to allosterically-induced receptor changes (Reid et al. 2001; Scheller et al. 1998). Indeed, studies with the Slp gene enhancer show that increased AR activity is due to the interplay of several weak AREs, and that N/C-interactions contribute to this effect (Scheller et al. 1998). Cooperative interactions of multiple AREs have also been reported in the Prostate-Specific Antigen (PSA) and probasin gene promotors (Reid et al. 2001). The demonstration of cooperativity between response elements suggests the existence of binding proteins able to recognize synergy control motifs and to bridge receptor dimers bound to adjacent DNA elements. A specific amino acid motif has been postulated to be responsible for the synergy control mechanism in the GR (Iniguez-Lluhi et al. 2000). Later on, they were identified as sumoylation sites. For the AR, the sumoylation sites in the AR-NTD have been proposed as synergy control motifs (Callewaert et al. 2004; Poukka et al. 2000), but the exact nature of the bridging factor is still unknown. How exactly the sumoylation controls synergism is unclear, but for the AR it seems less pronounced on selective AREs (Callewaert et al. 2004), indicating a role of DNA binding.

3.2 DBD-LBD Communication

Many AR mutations have been found in patients with complete or partial androgen insensitivity as well as in biopsies of castration-resistant metastatic prostate cancer

(Tilley et al. 1996). Most of these mutations affect the function of the domain they are situated in. However, some DBD mutations do not affect DNA binding and some LBD mutations do not affect ligand binding. Surprisingly, a DBD mutation can affect ligand binding and, vice versa, an LBD mutation can affect DNA binding (Helsen et al. 2012a). The mutated residues are situated at the surface of these domains pointing away from the DNA or the ligand. Based on modeling of the AR domains on the DBD-LBD coordinates of the crystal structure of the PPARγ-RXRα, as well as on docking AR-DBD against AR-LBD, a functional interface has been proposed between these domains, allowing signals from the DNA to reach the LBD and signals from the ligand to reach the DBD (Helsen et al. 2012a).

3.3 DBD-NTD Communication

The NTD is structurally important for AR-dependent gene expression by mediating multiple protein-protein interactions (McEwan 2004). In contrast to the LBD and the DBD, the NTD has little stable secondary structure (Reid et al. 2002). However, in vitro experiments with a purified AR-NTD-DBD polypeptide show that binding to an ARE results in changes in the intrinsic fluorescence emission spectrum of the NTD, indicating a conformational change in this domain upon DNA binding (Brodie et al. 2005). Moreover, protease-resistance of the polypeptide is increased in the presence of the DNA element, adding evidence for a conformational change in the NTD upon binding to the ARE (Brodie et al. 2005). Taken together, these findings indicate that DNA binding results in an allosterically-induced conformational change within the NTD, suggesting a communication between the DBD and the NTD.

3.4 Differential Effect of Selective Versus Classical AREs

Several features of the AR have been studied by monitoring the effect of point mutations on the functionality of the receptor in reporter assays. The effects of disrupting the N/C interaction, sumoylation of the NTD and the role of the polyglutamine stretch in the control of the overall activity of the human AR have initially all been described on reporter genes controlled by classical AREs (Claessens et al. 2008). However, the same analyses performed with reporter genes based on selective AREs gave much less pronounced or no effects. Indeed, deletion of the FQNLF motif in the NTD, which blunts the N/C-interaction, strongly reduced the AR capacity to transactivate through classical AREs but did not affect its activity on selective elements (Callewaert et al. 2003b). Mutation of lysine 385 in the NTD, blocking sumoylation at that site, clearly affects the cooperativity of the receptor on multiple classical AREs. However, when selective AREs where tested, the lysine 385 mutation did not increase the androgen response (Callewaert et al. 2004). Deletion of the polyglutamine tract results in an increase in the transactivation through classical

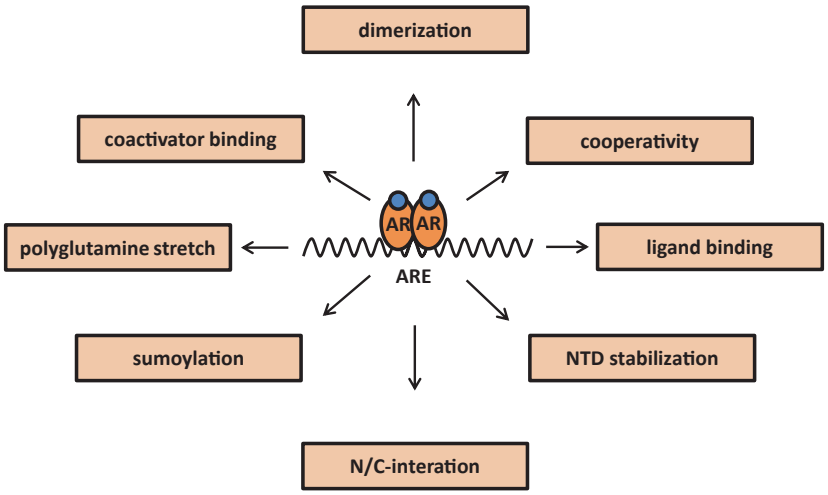


Fig. 3 Androgen response elements as allosteric modulators. The role of an androgen response element (*ARE*) is more complex than merely recruiting the androgen receptor (*AR*) to gene regulatory regions. Indeed, binding of the *AR* to AREs induces conformational changes, not only in the DBD but also in more distal regions, thereby influencing a variety of receptor functions such as dimerization, ligand and coactivator binding, and amino/carboxy-terminal (N/C)-interaction. Furthermore, the nature of the *ARE*, i.e. classical versus selective, allosterically influences the effect of several features on transactivation such as the length of the polyglutamine stretch and the sumoylayion status of the amino-terminal domain (*NTD*)

AREs, whereas this effect is not observed on selective elements (Callewaert et al. 2003a). Finally, modulation of AR activity by coactivators, AR sensitivity to proteolysis, and the effect of mutations in the AR dimerization interface of the DBD also vary with the nature of the ARE, the observed effect being different when using classical AREs compared to selective ones (Geserick et al. 2003).

Clearly, these data demonstrate that the DNA is not a passive partner of the AR, but influences several features of its activity (Fig. 3).

4 Conclusions

The AR was cloned more than 20 years ago (Lubahn et al. 1988). We have learned a lot about its main mechanisms of actions since then. However, we also know that there is still a lot to be discovered, even if we focus on the DNA binding alone. How can the DBD and the CTE control the different functions of the AR, and how can different DNA sequences affect the activity of the AR? Is there a direct interaction between the DBD and other domains? Despite strong indications, this still needs to be proven in structural analyses. Can we exploit the allosteric signals between the DBD and the other domains and translate them in therapeutic targets? And finally, it will be exciting to unravel the exact role of selective AREs in prostate cancer, and

in the control of the cell cycle in the primary tumor as well as in the metastases, be it hormone-sensitive or castration-resistant.

Acknowledgments We are grateful to all members of the Molecular Endocrinology Laboratory for constructive remarks and fruitful discussions. V.D. and M.L. are holders of a Doctoral Fellowship of the Flemish Fund for Scientific Research (FWO). L.C. is holder of a Doctoral Fellowship of the 'Agentschap voor Innovatie door Wetenschap en Technologie' (IWT). C.H. is supported by a grant of 'The L'Oréal-Unesco Fellowship for Young Women in Science' and of the FWO. This work was possible thanks to research grants from the FWO (G.0858.11 N) and from the KU Leuven (OT/11/081, OT/09/035).

References

Alen P et al (1999) The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 19:6085–6097

Bolton EC et al (2007) Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev 21:2005–2017

Brodie J et al (2005) Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. J Mol Endocrinol 34:603–615

Callewaert L et al (2003a) Implications of a polyglutamine tract in the function of the human androgen receptor. Biochem Biophys Res Commun 306:46–52

Callewaert L et al (2003b) Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements. J Biol Chem 278:8212–8218

Callewaert L et al (2004) Differential effect of small ubiquitin-like modifier (SUMO)-ylation of the androgen receptor in the control of cooperativity on selective versus canonical response elements. Mol Endocrinol 18:1438–1449

Cato AC et al (1987) The hormone response element of the mouse mammary tumour virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J 6:363–368

Claessens F et al (1989) Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein. Biochem Biophys Res Commun 164:833–840

Claessens F et al (1996) The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. J Biol Chem 271:19013–19016

Claessens F et al (2001) Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. J Steroid Biochem Mol Biol 76:23–30

Claessens F et al (2004) DNA recognition by nuclear receptors. Essays Biochem 40:59–72

Claessens F et al (2008) Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Recept Signal 6:e008

Cleutjens KB et al (1996) Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 271:6379–6388

Clinckemalie L et al (2013) Androgen regulation of the TMPRSS2 gene and the effect of a SNP in an androgen response element. Mol Endocrinol 27:2028–2040

De Bruyn R et al (2011) Identification and characterization of androgen response elements. Methods Mol Biol 776:81–93

Denayer S et al (2010) The rules of DNA recognition by the androgen receptor. Mol Endocrinol 24:898–913

Dubois V et al (2012) Androgens and skeletal muscle: cellular and molecular action mechanisms underlying the anabolic actions. Cell Mol Life Sci 69:1651–1667
Freedman LP (1992) Anatomy of the steroid receptor zinc finger region. Endocr Rev 13:129–145
Geserick C et al (2003) Differential modulation of androgen receptor action by deoxyribonucleic acid response elements. Mol Endocrinol 17:1738–1750
Grontved L et al (2013) C/EBP maintains chromatin accessibility in liver and facilitates glucocorticoid receptor recruitment to steroid response elements. EMBO J 32:1568–1583
Grosse A et al (2012) Androgen receptor-mediated gene repression. Mol Cell Endocrinol 352:46–56
Haelens A et al (2007) The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor. Cancer Res 67:4514–4523
Helsen C et al (2012a) Evidence for DNA-binding domain–ligand-binding domain communications in the androgen receptor. Mol Cell Biol 32:3033–3043
Helsen C et al (2012b) Structural basis for nuclear hormone receptor DNA binding. Mol Cell Endocrinol 348:411–417
Hurtado A et al (2011) FOXA1 is a key determinant of estrogen receptor function and endocrine response. Nat Genet 43:27–33
Ikonen T et al (1997) Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem 272:29821–29828
Iniguez-Lluhi JA et al (2000) A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol Cell Biol 20:6040–6050
Irvine RA et al (2000) Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. Hum Mol Genet 9:267–274
Jia L et al (2008) Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. PLoS One 3:e3645
John S et al (2011) Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. Nat Genet 43:264–268
Kerkhofs S et al (2012) A role for selective androgen response elements in the development of the epididymis and the androgen control of the 5alpha reductase II gene. FASEB J 26:4360–4372
Kotaja N et al (2002) PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol 22:5222–5234
Lanzino M et al (2010) Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells–identification of a novel androgen response element. Nucleic Acids Res 38:5351–5365
Laurent M et al (2013) Osteoporosis in older men: recent advances in pathophysiology and treatment. Best Pract Res Clin Endocrinol Metab 27:527–539
Lubahn DB et al (1988) Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240:327–330
Lupien M et al (2009) Cistromics of hormone-dependent cancer. Endocr Relat Cancer 16:381–389
Ma H et al (1999) Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol Cell Biol 19:6164–6173
McEwan IJ (2004) Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. Endocr Relat Cancer 11:281–293
McEwan IJ et al (2007) Natural disordered sequences in the amino terminal domain of nuclear receptors: lessons from the androgen and glucocorticoid receptors. Nucl Recept Signal 5:e001
Parker MG et al (1987) Identification of androgen response elements in mouse mammary tumour virus and the rat prostate C3 gene. J Cell Biochem 35:285–292
Payvar F et al (1983) Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35:381–392
Poukka H et al (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci USA 97:14145–14150
Qi W et al (2012) Negative androgen-response elements mediate androgen-dependent transcriptional inhibition of TGF-beta1 and CDK2 promoters in the prostate gland. J Androl 33:27–36
Reid KJ et al (2001) Two classes of androgen receptor elements mediate cooperativity through allosteric interactions. J Biol Chem 276:2943–2952

Reid J et al (2002) Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions. J Biol Chem 277:20079–20086

Roche PJ et al (1992) A consensus DNA-binding site for the androgen receptor. Mol Endocrinol 6:2229–2235

Sahu B et al (2011) Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer. EMBO J 30:3962–3976

Sahu B et al. (2014). Androgen receptor uses relaxed response element stringency for selective chromatin binding and transcriptional regulation in vivo. Nucleic Acids Res: accepted for publication.

Schauwaers K et al (2007) Loss of androgen receptor binding to selective androgen response elements causes a reproductive phenotype in a knockin mouse model. Proc Natl Acad Sci USA 104:4961–4966

Scheller A et al (1998) Multiple receptor domains interact to permit, or restrict, androgen-specific gene activation. J Biol Chem 273:24216–24222

Schoenmakers E et al (1999) Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochem J 341(3):515–521

Schoenmakers E et al (2000) Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses. J Biol Chem 275:12290–12297

Shaffer PL et al (2004) Structural basis of androgen receptor binding to selective androgen response elements. Proc Natl Acad Sci USA 101:4758–4763

So AY et al (2007) Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. PLoS Genet 3:e94

Takayama K et al (2007) Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis. Oncogene 26:4453–4463

Tanner TM et al (2010) A 629RKLKK633 motif in the hinge region controls the androgen receptor at multiple levels. Cell Mol Life Sci 67:1919–1927

Taslim C et al (2012) Integrated analysis identifies a class of androgen-responsive genes regulated by short combinatorial long-range mechanism facilitated by CTCF. Nucleic Acids Res 40:4754–4764

Tewari AK et al (2012) Chromatin accessibility reveals insights into androgen receptor activation and transcriptional specificity. Genome Biol 13:R88

Tilley WD et al (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res 2:277–285

Tsai MJ et al (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486

Umesono K et al (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 57:1139–1146

Verrijdt G et al (2000) Change of specificity mutations in androgen-selective enhancers. Evidence for a role of differential DNA binding by the androgen receptor. J Biol Chem 275:12298–12305

Verrijdt G et al (2003) Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. Mol Genet Metab 78:175–185

Verrijdt G et al (2006) The androgen receptor DNA-binding domain determines androgen selectivity of transcriptional response. Biochem Soc Trans 34:1089–1094

Wang Q et al (2005) Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 19:631–642

Wang Q et al (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 27:380–392

Zhang M et al (1997) Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci USA 94:5673–5678

Allosteric Regulation and Intrinsic Disorder in Nuclear Hormone Receptors

Jordan T. White, Hesam N. Motlagh, Jing Li, E. Brad Thompson and Vincent J. Hilser

1 Dynamics and Intrinsic Disorder in NHRs

One of the most rapidly expanding fields in protein biophysics is the study of intrinsic disorder in proteins (Tompa 2011). In the past decade, it was realized that these intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) are hyper-abundant in eukaryotic transcription factors (Liu 2006). As a major class of transcription factors, NHRs are no exception, and all NHRs show some disorder propensity (Krasowski et al. 2008). Most NHRs are multidomain proteins consisting of ligand-binding (LBD), DNA-binding (DBD) and often, N-terminal (NTD) domains (Fig. 1).

The LBD contains several dynamic regions that are predominantly near the ligand-binding pocket. Some of the earliest research on the dynamic nature of the LBD is from the O'Malley lab in the 1990's. Working with progesterone and estrogen receptors, they found that the LBD undergoes a conformational change upon binding hormone (Allan et al. 1992a, 1992b; Beekman et al. 1993). Subsequent X-ray crystallography and fluorescence anisotropy experiments demonstrated that when the LBD binds ligand, helix-12 undergoes a conformational change and becomes less dynamic (Bourguet et al. 1995; Renaud et al. 1995; Wagner et al. 1995; Kallenberger et al. 2003). This conformational change varies depending on the ligand bound; hence different ligands can promote specific conformations that bind corepressors or coactivators (Brzozowski et al. 1997; Nahoum et al. 2007).

V. J. Hilser (✉) · J. T. White
Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA
e-mail: hilser@jhu.edu

H. N. Motlagh · J. Li · V. J. Hilser
T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD 21218, USA

E. B. Thompson
Center for Nuclear Receptors and Cell Signaling, Department of Biology and Biochemistry, University of Houston, Houston, TX 77204-5056, USA

Prof. Emeritus, University of Texas Medical Branch, Galveston, TX, USA

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_5

Fig. 1 The domain organization of NHR's. The diagram reads N-terminus on the left, C-terminus on the right. The N-terminal domain (*NTD*) is intrinsically disorder and often binds cofactor proteins. The DNA-binding domain (*DBD*) binds response elements. The ligand-binding domain (*LBD*) binds small molecules, such as steroids, and some cofactor proteins. The *NTD* length is highly variable and its sequence is poorly conserved. The *DBD* and *LBD* together are on the order of 300 amino acids and are well conserved

Researchers have also shown that cofactor fragments that bind the LBD can stabilize the LBD interaction with certain ligands (Gee et al. 1999; Kallenberger et al. 2003), demonstrating an allosteric communication between cofactor and ligand binding. The cofactor thus acts as an allosteric effector/ligand.

The DBD also has regions that are dynamic. Estrogen and glucocorticoid receptor DBDs are partially disordered in solution (Berglund et al. 1997; Schwabe et al. 1990) , and become more ordered when bound to DNA (Schwabe et al. 1995; Luisi et al. 1991). Spolar and Record first described this transition on thermodynamic grounds (Spolar and Record 1994), and their analysis hinted at the possibility of different DNA sequences having different effects on the DBD. Indeed, the sequence of DNA does modulate this conformational change (Bain et al. 2012; Meijsing et al. 2009; Loven et al. 2001). Specifically, the DNA sequence can change both the binding affinity to the DBD and the propensity for dimerization of the DBD (Hudson et al. 2013; Wood et al. 2001; Glass et al. 1988). It has been speculated that response elements cause allosteric effects beyond the DBD—possibly changing the conformation of the NTD (Lefstin et al. 1994; Starr et al. 1996), a point that will be revisited in the next section.

The steroid hormone receptor (SHR) subclass of NHRs contains large NTDs of varying size and sequence, which are perhaps the best example of dynamics and disorder in the NHR family of proteins (Krasowski et al. 2008). Each SHR type contains a large amount of ID in the N-terminus, as determined by a myriad of biophysical methods (Fischer et al. 2010; Dahlman-Wright et al. 1995; Kumar and Thompson 2010; Bain et al. 2000, 2001; Lavery and McEwan 2008; Nocula-Ługowska et al. 2009). It is well-established that many disordered proteins undergo coupled folding and binding to perform their biological role (Uversky et al. 2005). In 1999, two manuscripts presented the first evidence that the NTD of an SHR can fold into a tertiary structure, either because of the DBD binding a response element or because of high concentrations of an osmolyte (Baskakov et al. 1999; Kumar et al. 1999). When so folded, the NTD showed enhanced binding of known partner proteins (Kumar et al. 2001). Since then, many groups have used various osmolytes, such as trimethyl amine-N-oxide (TMAO; see Bolen and Baskakov 2001) to fold the NTDs of NHRs and have demonstrated that they fold cooperatively, a hallmark of a naturally evolved protein (Watters et al. 2007).

Glucocorticoid, androgen, and mineralocorticoid receptors' NTDs all fold cooperatively in the presence of molar concentrations of TMAO (Reid et al. 2002;

Li et al. 2012; Fischer et al. 2010; Baskakov et al. 1999). This is evidence of a major folded conformation or an ensemble of folded conformations. The folded state is the presumed biologically active state of the NTD, and this is supported by studies showing the NTD folds upon binding transcriptional cofactors. The NTDs of SHRs bind numerous cofactor proteins (Lavery and McEwan 2005). Studies on estrogen, glucocorticoid, and androgen receptors have shown that the conformational ensembles of the NTDs become more ordered upon binding cofactor proteins (Reid et al. 2002; Copik et al. 2006; Warnmark et al. 2001; Khan et al. 2012; Kumar et al. 2004). This suggests a coupled folding and binding mechanism that regulates SHR activity.

In sum, all NHR domains—even the relatively structured LBDs and DBDs—are dynamic and exist as conformational ensembles of states. Upon binding a ligand, domains are stabilized in one or another globular state of a more limited ensemble. These ligand-specific effects have functional consequences on the co-regulators bound, and on transcriptional function. By varying the transcriptional function of a receptor, ligands can alter cellular and clinical outcomes. "Ligand" thus refers to all binding partners—protein, DNA, steroidal or other small molecules—as they interact with their respective domains.

2 Inter- and Intra-Domain Coupling in NHRs

Binding of a ligand in one NHR domain not only affects the conformational ensemble of that domain, but also the conformational ensembles of the other domains. This allosteric coupling between and within the major domains is crucial for the function of the receptors.

2.1 DNA Response Elements as Allosteric Effectors

Early glucocorticoid research raised a fundamental question (Ivarie and O'Farrell 1978)—how does a NHR activate a multitude of genes to different degrees? One possibility is that each response element could modify the activity of a bound NHR. This idea was supported by research on glucocorticoid, estrogen, and thyroid hormone receptors (Lefstin et al. 1994; Allan et al. 1992b; Glass et al. 1988; Starr et al. 1996; Sakai et al. 1988; Martinez and Wahli 1989). In particular, work from Yamamoto and colleagues demonstrated that glucocorticoid receptor activity was dependent on the response element sequence (Sakai et al. 1988) and also speculated that a conformational change was occurring upon DNA binding.

Ikeda and colleagues provided the first evidence of different response elements inducing different conformational changes in NHRs (Ikeda et al. 1996). They found that transcriptionally active response elements induced a change in thyroid hormone/retinoid X receptor dimers, such that the complex was resistant to protease

digestion, relative to protein dimers on inactive response elements (Ikeda et al. 1996). Later studies found similar effects with estrogen and androgen response elements and their cognate receptors (Wood et al. 1998, 2001; Geserick et al. 2003; Loven et al. 2001).

The limited proteolysis experiments described above demonstrate DNA response element dependent protection patterns. However, which portions of the molecule were undergoing conformational changes was still an open question. In 1999, Kumar and colleagues published the first manuscript demonstrating that when an NHR, glucocorticoid receptor, binds a site-specific DNA its NTD undergoes a conformational change (Kumar et al. 1999). Kumar used a combination of circular dichroism and tryptophan fluorescence to demonstrate that folding was occurring in the NTD. Subsequent studies have revealed similar phenomena in progesterone receptor (Bain et al. 2000, 2001), estrogen receptor alpha (Greenfield et al. 2001), and androgen receptor (Brodie and McEwan 2005). The inferred significance of these observations is that binding of DNA coupled to folding of the NTD would recruit transcriptional cofactors, as proposed by Thompson and coworkers (Copik et al. 2006; Thompson and Kumar 2003).

2.2 LBD Ligands as Allosteric Effectors

Different ligands binding to the LBD can elicit specific transcriptional responses. Early results on this matter are conflicting. Several studies suggested that binding of hormone increased the binding affinity for DNA (Bagchi et al. 1988; Yamamoto and Alberts 1972; Becker et al. 1986) while others did not (King and Greene 1984; Welshons et al. 1984). Some of these ambiguities are likely due to different DNA response elements used by different labs. As shown by Meyer and coworkers, different response elements can modulate the effect of a given hormone. They demonstrated that RU486, a known antagonist, of the progesterone receptor A isoform could activate the B isoform. Furthermore, this activation only occurred on one of the two promoter sequences that they tested (Meyer et al. 1990). This is an example of selective response modification by an NHR, in which a ligand modifies receptor activity in a manner dependent on the DNA bound (Gerber et al. 2009). By this and probably other mechanisms, selective sets of genes are activated or repressed by specific steroids acting through their cognate receptors. Such ligands activate distinct, but usually overlapping sets of genes.

Selective response modifiers exert their effects through a number of mechanisms. Besides affecting DNA binding, ligands also change subsequent events. Different ligands alter recruitment of NTD binding partners to estrogen and glucocorticoid receptors (Ronacher et al. 2009; Garside et al. 2004; Shang and Brown 2002). Furthermore, ligands can also alter dimerization of NHRs, as seen in the formation of PPARα-RXRα heterodimers on DNA (Forman et al. 1997). Regardless of the variety of mechanisms, what is clear from these examples is that ligand binding at the LBD causes allosteric effects on the DBD and NTD.

2.3 *Splicing and Translational Isoforms Modulate Allosteric Communication*

As do many proteins, NHRs can exist in multiple isoforms (Lu and Cidlowski 2005; Cao et al. 2013; Tontonoz et al. 1994; Schrader and O'Malley 1972; Talbot et al. 1993), and several studies have demonstrated that NHR isoforms differ in their transcriptional activities (Mouillet et al. 2001; Lu and Cidlowski 2005; Meyer et al. 1990). However, it is rarely made clear how isoforms have different transcriptional activities or specificities. One possibility is that each isoform contains a unique system of allosteric coupling and disorder. This notion is supported by recent work showing that tissue-specific coding exons are enriched in intrinsic disorder and binding motifs (Buljan et al. 2012). Interestingly, NTDs of NHRs are often intrinsically disordered and full of co-regulator binding sites (Krasowski et al. 2008). This raises the possibility that tissues tune the activity of a NHR by expressing varying amounts of the NTD isoforms.

Three steroid receptors are known to possess alternative NTD isoforms (progesterone, glucocorticoid, and ecdysone receptors; see: Kastner et al. 1990; Lu and Cidlowski 2005; Talbot et al. 1993). Of the three ecdysone receptor isoforms, two have very similar NTD lengths and yet vastly different effects on transcriptional activity (Mouillet et al. 2001; Dela Cruz et al. 2000). Similar effects have been reported for progesterone and glucocorticoid receptors (Meyer et al. 1990; Lu and Cidlowski 2005), and in the case of glucocorticoid receptor this change in NTD length has an effect on the folding free energy of this disordered domain (Li et al. 2012). Interestingly, the isoform with the lowest folding energy is transcriptionally the most active. This is most readily explained by coupled folding of NTD with binding of coregulators because a more stable isoform will bind to coregulators more strongly.

LBD isoforms also occur. For example, the glucocorticoid receptor has three exon splice-variant isoforms that alter the LBD length (Russcher et al. 2007). Of these, only GRα is able to bind hormone. The other two (β and γ) have truncated LBDs and markedly different effects on transcriptional regulation (de Lange et al. 2001; Russcher et al. 2005). Mechanistically, the shorter LBDs ablate the ability of hormone to induce a change in the receptor. However, how these changes affect LBD allosteric coupling to the other receptor domains has not been studied in detail.

In sum, NHRs use inter- and intra-domain coupling to govern their responses to a variety of allosteric effector ligands and thus determine the specificity of their function. Binding of its ligand at any given domain (NTD, DBD, LBD) can affect the stability and binding affinity of their ligands at other domains. These allosteric changes have been understood from a phenomenological view for many years. Only recently has a model been developed that explains these phenomena in testable, quantitative terms. Below we will discuss this general model of allostery (the Ensemble Allosteric Model) that can be used to glean mechanistic insight into NHR allostery.

3 The Ensemble Allosteric Model: Application in the Case of NHRs

How allostery works is a century-old question (Barcroft and Hill 1910; Adair 1925) that was initially addressed with hemoglobin. Monod, Wyman, and Changeux proposed arguably the most influential allosteric model using macroscopic, thermodynamic concepts to take into account a conformational change within the subunits of hemoglobin (Monod et al. 1965). Other influential models have been proposed that explain most of the available data on hemoglobin (Koshland et al. 1966; Henry et al. 2002). However, all these models have limitations as they are phenomenological and do not address "how" allostery is mediated between distal sites (Motlagh et al. 2012). Crystallographic or other structural data has been used to elucidate bond paths linking the binding site of the allosteric effector with a distant responsive site (Perutz et al. 1987; Perutz et al. 1998; M.F. 1970; Suel et al. 2003; Lockless and Ranganathan 1999). Even though it has been suggested that these allosteric pathways may be dominant (Daily and Gray 2009), they do not explain the following: all of the available data on hemoglobin (Smith and Ackers 1985), proteins in which no bond path can be found between the allosteric effector binding site and the distant response region, or the perplexing observation of protein dynamic- and disorder- mediated allostery (Petit et al. 2009; Ferreon et al. 2013; Reichheld et al. 2009).

We recently proposed a model that alleviates some of these issues by articulating allostery in terms of the intrinsic energetics of a protein—the Ensemble Allosteric Model (EAM) (Hilser and Thompson 2007; Motlagh and Hilser 2012; Hilser et al. 2012; Motlagh et al. 2012). Because it applies to all proteins, the EAM can be used to describe allostery in NHRs. In particular, it can explain perplexing phenomena, such as the ability of a single ligand to be an agonist or an antagonist to NHR function in different cells (Wolf and Jordan 1992; Amsterdam et al. 2002).

The EAM is grounded in two well-established observations from protein allostery: The ability of allosteric proteins to exist in multiple conformations in solution, and their segregation of binding sites into different domains. Such domains can communicate with one another, the essence of allostery. From these simple and well-established facts, it is possible to develop the model and ask quantitative questions about allostery.

Consider an allosteric protein consisting of three interacting domains (Fig. 2). The simplest conformational ensemble of each domain is a two state equilibrium between at least one high affinity (H) and low affinity (L) state for its ligand. The L state of a domain can be either intrinsically disordered or an ensemble of somewhat ordered conformers that bind its ligand with lower affinity than does the H conformation. The H and L states for each domain have a free energy difference (i.e. ΔG_i), which determines how often the molecule is in the H or L conformation in the absence of influences from other domains. Because the domains communicate to one another, there must be an interaction energy between them. When one domain goes to its L conformation it either stabilizes (i.e. $\Delta g_{1,2} < 0$) or destabilizes (i.e. $\Delta g_{1,2} > 0$)

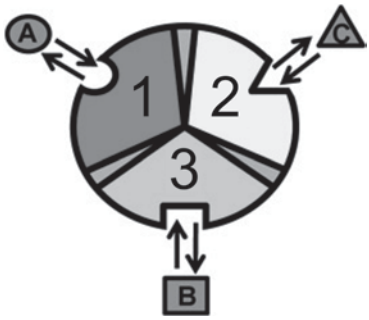


Fig. 2 Cartoon of an allosterically coupled protein with three subunits. The three subunits (1, 2, 3) bind to one ligand each (*A*, *B*, *C*). In the context of NHR's, the three subunits are the NTD, DBD, and LBD binding to cofactor, DNA, and ligand, respectively. Taken from (Motlagh and Hilser 2012) with permission

the H state of the other domain(s) to which it is coupled (Fig. 2 and Table 1). From this simple articulation, enumeration of all combinations of H and L domain states in addition to their relative free energies is straightforward. Table 1 lists every possible combination of domains 1, 2, and 3 being in either the H or L conformation. The free energy of each state is simply the sum of the conformational energy and the interaction energy. Taking the statistical weight of each state from Table 1 and summing it, yields the partition function (Q) which gives access to every quantity of interest, including experimental observables:

$$Q = \sum_j S_j = S_{HHH} + S_{HHL} + S_{HLH} + S_{LHH} + S_{HLL} + S_{LLH} + S_{LHL} + S_{LLL}$$

With this, it is possible to reproduce basic allosteric phenomena and ask basic questions. For instance, what happens when ligand A is introduced? The H conformation of each domain will preferentially bind its proper ligand at its introduction into the system (Wyman and Gill 1990), and introduction of ligand A will stabilize each microstate in Table 1 that has domain 1 in the H conformation by a free energy of:

$$\Delta g_{Lig\ A} = -RTln(1 + K_{a,A}[A]) = -RTln(Z_{Lig,A})$$

In turn, this will change the partition function:

$$Q_{w/A} = Z_{Lig,A}(S_{HHH} + S_{HHL} + S_{HLH} + S_{HLL}) + S_{LHH} + S_{LLH} + S_{LHL} + S_{LLL}$$

where $Z_{Lig,A}$ acts as a weighting term that takes into account the increase in the probability of the H conformation of domain 1 in the presence of A (i.e. $Z_{Lig,A} = 1 + K_a*[A]$). Note that at A = 0 this equation reduces to the original partition function.

Ligand binding to domain 1 changes the statistical weight of some states, but in turn this will change the probability of all states. Of particular interest is the change in probability of the H state of an "active site" (Domain 3; e.g. the binding site for a cofactor protein in the NTD). The probability of domain 3 being in the high affinity, active state without A present is simply the statistical weight of states where domain 3 is in the H state divided by the partition function:

Table 1 Breakdown of allosteric states and energies

State	$\Sigma\Delta G_i$	$\Sigma\Delta \mathbf{g}_{i,j}$	S_i	Probability
HHH	0	0	1	S_{HHH}/Q
LHH	ΔG_1	$\Delta g_{12}+\Delta g_{13}$	$K_1\phi_{12}\phi_{13}$	S_{LHH}/Q
HLH	ΔG_2	$\Delta g_{12}+\Delta g_{23}$	$K_2\phi_{12}\phi_{23}$	S_{HLH}/Q
HHL	ΔG_3	$\Delta g_{13}+\Delta g_{23}$	$K_3\phi_{13}\phi_{23}$	S_{HHL}/Q
HLL	$\Delta G_2+\Delta G_3$	$\Delta g_{12}+\Delta g_{13}+\Delta g_{23}$	$K_2K_3\phi_{12}\phi_{13}\phi_{23}$	S_{HLL}/Q
LHL	$\Delta G_1+\Delta G_3$	$\Delta g_{12}+\Delta g_{13}+\Delta g_{23}$	$K_1K_3\phi_{12}\phi_{13}\phi_{23}$	S_{LHL}/Q
LLH	$\Delta G_1+\Delta G_2$	$\Delta g_{12}+\Delta g_{13}+\Delta g_{23}$	$K_1K_2\phi_{12}\phi_{13}\phi_{23}$	S_{LLH}/Q
LLL	$\Delta G_1+\Delta G_2+\Delta G_3$	$\Delta g_{12}+\Delta g_{13}+\Delta g_{23}$	$K_1K_2K_3\phi_{12}\phi_{13}\phi_{23}$	S_{LLL}/Q

This table is modified from (Motlagh and Hilser 2012). $\Delta\mathbf{G_i}$ is the free energy difference between the high (*H*) affinity and the low (*L*) affinity state of domain i. $\Delta\mathbf{g_{i,j}}$ is the energy of interaction between two subunits, i and j. $\mathbf{S_i}$ is the statistical weight for a given state and Q is the sum of all statistical weights: $S_i = e^{\frac{-(\sum \Delta G_i + \sum g_{i,j})}{RT}}$

$$P_{3,H}([A]=0) = \frac{S_{HHH} + S_{HLH} + S_{LHH} + S_{LLH}}{Q}$$

With A present all states with domain 1 in the H state will be redistributed:

$$P_{3,H}([A]>0) = \frac{Z_{Lig,A}(S_{HHH} + S_{HLH}) + S_{LHH} + S_{LLH}}{Q_{w/A}}$$

Upon binding domain 1, ligand A will redistribute the ensemble and will either increase the probability of domain 3 being active or decrease the probability of domain 3 being active. To relate the change in probability to the amount of energetic perturbation, we may define a value called the Coupling Response (CR) (Hilser and Thompson 2007) which is the change in probability of a state normalized to the energy of ligand binding:

$$CR_{3,A} = \frac{\Delta P_{3,H}}{\Delta \ln(Z_{Lig,A})} = \frac{P_{3,H}([A]>0) - P_{3,H}([A]=0)}{\ln(Z_{Lig,A})}$$

A positive CR indicates that ligand A binding to its own domain increases the probability of domain 3 being in the active or H conformation. The opposite is true for negative CR values, which represent negative effects on the stability of a domain (Fig. 3).

In this three-domain protein example, one can consider the effect of how an additional ligand, one that binds domain 2 affects the allosteric response of domain 3. When ligand B binds domain 2 it can change the magnitude of the response of domain 3 to ligand A bound in domain 1. The coupling response of other domains to ligand A:Domain 1 binding can change from positive to negative and visa-versa.

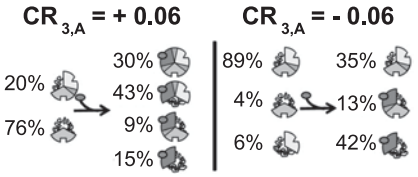


Fig. 3 Examples of CR for single ligand binding. A positive $CR_{3,A}$ indicates that binding of ligand A to domain 1 stabilizes states where domain 3 is in its high affinity conformation. Domains 1, 2, and 3 are displayed as the top left, bottom, and top right of the circle, respectively. The parameters of the positive $CR_{3,A}$ are: $\Delta G_1 = -1.7$, $\Delta G_2 = 2.0$, $\Delta G_3 = -0.9$, $\Delta g_{12} = -2.3$, $\Delta g_{23} = 0.1$, $\Delta g_{13} = 1.5$, and $\Delta g_{Lig,A} = -5.0$ in kcal/mol. The parameters of the negative $CR_{3,A}$ are: $\Delta G_1 = -2.1$, $\Delta G_2 = 1.0$, $\Delta G_3 = 1.2$, $\Delta g_{12} = -1.7$, $\Delta g_{23} = 0.6$, $\Delta g_{13} = -2.7$, and $\Delta g_{Lig,A} = -5.0$ in kcal/mol. From (Motlagh and Hilser 2012) with permission

In a similar manner as before, we can consider the CR of domain 3 when ligand A is added while ligand B is already present:

$$CR_{3,A}(B>0) = \frac{P_{3,H}([A]>0 \mid B>0) - P_{3,H}([A]=0 \mid B>0)}{\ln(Z_{Lig,A})}$$

Note that this equation takes into account the effect of ligand B by itself, thus the CR being described tells us how ligand B changes the allosteric response to ligand A. With two ligands present we can begin to see some combinations of parameters exist such that ligand A can act as either a positive or negative regulator of domain 3, a paradoxical observation that has been noted in the NHR field and is discussed in the following section (Fig. 4).

4 The Ensemble Allosteric Model may Reconcile Puzzling Observations in NHRs

The above model is general and applicable to all allosteric systems, including SHRs. Each of the three domains of an SHR has binding sites for multiple binding partners, similar to the articulation of the EAM. The C-terminal LBD binds small steroidal and synthetic ligands; it also binds a variety of co-regulatory proteins. The central DBD binds DNA sequences and other proteins; DNA acts as a ligand and can have sequence specific effects on transcription and DBD stability. The NTD also binds co-regulators. Application of the EAM to the SHRs at once makes it apparent that all these non-SHR binding partners must be considered ligands, as the preceding discussion explains. Further, the EAM obviously also applies to the two domain group of NHRs (Hilser and Thompson 2007).

The model has the potential to explain some perplexing observations in NHR research that have important implications for practical applications and drug development. Tamoxifen is known to inhibit breast cancer and yet promote uterine cancer

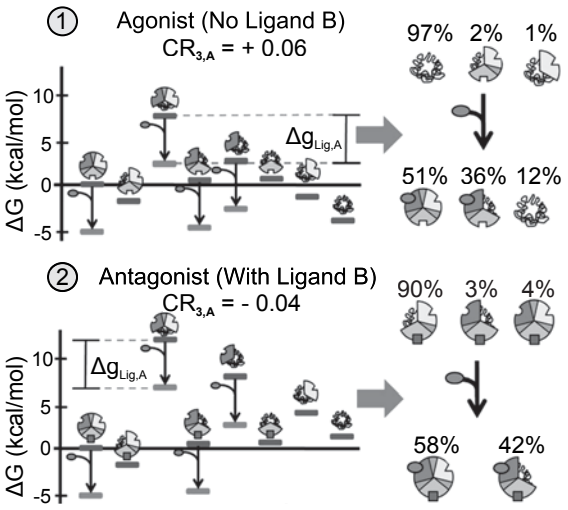


Fig. 4 Positive-Negative response switching. In case 1 ligand A acts as a positive regulator of domain 3. In case 2 the presence of ligand B causes ligand A to act as a negative regulator of domain 3. The parameters used: $\Delta G_1 = -6.75$, $\Delta G_{2,B=0}$ (case 1) $= -4.4$, $\Delta G_{2,B>0}$ (case 2) $= 0.6$, $\Delta G_3 = -2.7$, $\Delta g_{12} = 6.8$, $\Delta g_{23} = 4.8$, $\Delta g_{13} = -1.9$, and $\Delta g_{Lig,A} = -5.0$ kcal/mol. Domains 1, 2, and 3 are displayed as the top left, bottom, and top right of the circle, respectively. From (Motlagh and Hilser 2012) with permission

(Wolf and Jordan 1992), glucocorticoids exert cell-specific anti-inflammatory effects (Amsterdam et al. 2002), and a number of other NHR's have effects in a tissue or isoform specific manner (Truman et al. 1994; Walters et al. 2010; Han et al. 2006). Furthermore, many steroidal and non-steroidal ligands for the LBD act as selective response modifiers (SRMs), meaning that they alter the transcription of selected, though overlapping, sets of genes, in a cell-specific way.

These phenomena may be understood in terms of thermodynamic ensembles. In different cellular contexts, the equilibria of the ensemble could be tuned by perturbations including but not limited to: (1) type and accessibility of DNA response elements; (2) abundance and type of natural/synthetic ligands to LBD; (3) the type and abundance of cofactors to NTD and LBD; (4) different distribution of NHR splicing and translational isoforms; (5) different post-translational modifications to the NHR; (6) effects of intracellular pH, small ion and organic osmolyte levels. All of these will affect the energetic landscape of the ensemble.

As mentioned previously in this chapter, there are known allosteric effects of DNA sequence on the DBD and on NHR function (Bain et al. 2000; Meijsing et al. 2009; Ikeda et al. 1996; Wood et al. 2001). Binding to different gene elements may bias an NHR to bind certain cofactors. This represents a ligand-based effect on the thermodynamics of another domain. Since it has been shown for glucocorticoid and progesterone receptors that DBD binding of DNA results in acquisition of structure and function in the NTD, there is physical evidence to support this allosteric effect (Bain et al. 2000; Kumar et al. 1999; Kumar and Thompson 2010).

Recent results suggest that the response element itself is part of the concentration-limiting step of transcriptional induction (Blackford et al. 2012), thus gene elements may be a sensitive area of regulation. Gene elements could vary in

accessibility because of different DNA methylation patterns in tissues (for review Garcia-Carpizo et al. 2011), expression of different histones (Hake et al. 2006), or different post-translational modifications of histones (Hake et al. 2006). NHR actions do appear to be linked to DNA methylation and to histone modifications (Hsu et al. 2010; Kangaspeska et al. 2008; Nie et al. 2000), and how this affects NHR function is still an active area of research. Put together, there is a large repertoire of perturbations that can modulate the energetics of DNA binding and thus the macroscopic, biological effect.

Nature may also produce different NHR activities through LBD binding of structural variants of hormones (Norman et al. 2004; Diamanti-Kandarakis et al. 2009). Hormone:LBD binding affects both LBD and NTD stability and their subsequent binding of co-regulators. While the major steroid-producing glands are the source of most circulating steroid hormones, it is now clear that local, tissue-specific steroid synthesis and metabolism can cause the local concentration and type of steroid ligand to vary dramatically. In addition, cell and tissue variations of SHR isoforms and concentrations can vary. The level of SHR in a cell can shift the dose-response curve to its cognate ligand by up to an order of magnitude (Szapary et al. 1996, 1999). All of these different hormonal effects would change the probabilities in the conformational ensemble of the LBD. This in turn would affect the distribution of states for other domains of a given NHR.

Binding of different cofactors is a third way to vary NHR activity. There are a large number of NHR co-regulators (Aranda and Pascual 2001; Jenkins et al. 2001), and differential expression between tissues could cause variation in NHR response. It is worthwhile to note, however, that most NHR co-regulators appear to be rather general. Very few tissue specific co-regulators have been documented, an example: PGC-1 appears to exist exclusively in muscle, kidney, and liver cells (Knutti et al. 2000). Until further evidence of tissue specific co-regulators is identified, it must remain speculation that they are the chief explanation for selective response modifier effects. Moreover, coregulators do not act singly, but in large heteromeric collections that are bound to each other and to the NHR by one or a few “platform” coregulators. Cell-specific actions could be determined by the collective action of each heteromeric group. This in turn, would be driven by the presence and concentration of each coregulator (Blackford et al. 2012). The EAM shows how ligands could alter the choice or affinity for platform coregulators by positive or negative cooperativity within the NHR; thus, accounting for selective responses and even for cell/tissue-specific switching of agonist to antagonist.

Expression of different protein isoforms is a fourth possibility to explain different tissue activities of NHRs. Some NHRs have multiple isoforms of the ID N-terminus. Each N-terminal isoform may have a different intrinsic stability, which will result in a different sensitivity to cofactors (Li et al. 2012). Each isoform may also have different energies of interaction with the other domains. Simply expressing a different protein isoform could both change the sensitivity to NTD binding partners and the coupling response to allosteric regulators. Since NHRs act as homo- or hetero-dimers, heteromers of NHR isoforms may alter the net response to a given ligand.

Post-translational modifications of NHRs include phosphorylation (Dougherty et al. 1982; Pike and Sleator 1985; Housley and Pratt 1983), sumoylation (Le Drean et al. 2002; Poukka et al. 2000), ubiquitylation (Wallace and Cidlowski 2001; Adachi et al. 2002), and acetylation (Fu et al. 2000; Li et al. 2007). Each modification has the potential to change NHR turnover, binding of ligands, and/or the coupling between NHR domains. For example, certain GR NTD phosphorylations—known to alter its transcriptional activity—also stabilize the NTD (Webster et al. 1997).

Considering the net effect of this long list of influences, the EAM may lead to an understanding of how the paradox of cell-specific selective response to a single steroid ligand occurs. Our model also explains how a given steroidal ligand can act as an agonist in one cell and an antagonist in another. A scheme for the application of EAM follows: First, the intrinsic coupling of a protein must be determined. Most of the exact values need to be known, but EAM can be used to estimate a few values if their sign and order of magnitude are known. Next, our model can be used to simulate the probabilities of the high affinity (active) states. These simulations can be done with and without ligands, and the effect of mutations can also be tested. A given mutation could affect the allostery between two domains (Δg of interaction) and/or the intrinsic stability of a domain (ΔG of the domain). Note that in these simulations the high affinity state is proportional to protein activity, or transcriptional activity in the case of NHRs. Lastly, after simulating the results desired, one can test multiple mutations to find some that match the predicted outcome. Alternatively, if the interest is in drugs, then one can test multiple ligands for the desired coupling response.

5 Conclusions

The interplay between allostery and intrinsic disorder are just beginning to be unraveled for NHRs specifically, and transcription factors generally. It is clear thus far that these proteins use conformational dynamics to couple binding with allosteric effects. This potentially strengthens the cross talk between different receptor domains. Our ensemble allosteric model is a theoretical framework that describes how a change in ensembles could drive allosteric effects, and it gives testable predictions for how NHRs should couple to their ligands and to themselves. Using the coupling response, the ensemble allosteric model allows us to predict the biological effect of drugs and cofactor proteins that bind NHRs. Positive regulators of transcriptional activity will have a positive coupling response with NHR domains that mediate transcriptional activity, and the opposite is predicted for negative regulators. This simple observation produces testable predictions for allosteric drug design and targeting. It also yields predictions that connect in vivo assays, measuring transcriptional activity, to in vitro assays, measuring protein stability.

Acknowledgments VJH is supported by NIH grant GM063747 and NSF grant MCB-0446050. JTW was supported by the NIH training grant 5T32GM007231-39 to the Johns Hopkins University department of biology. HNM was supported by the NIH training grant T32-GM008403.

References

Adachi S, Okuno M, Matsushima-Nishiwaki R, Takano Y, Kojima S, Friedman SL, Moriwaki H, Okano Y (2002) Phosphorylation of retinoid X receptor suppresses its ubiquitination in human hepatocellular carcinoma. Hepatology 35(2):332–340. doi:10.1053/jhep.2002.31164

Adair GBAF, H Jr (1925) The hemoglobin system: VI. The oxygen dissociation curve of hemoglobin. J Biol Chem 63:529–545

Allan GF, Leng X, Tsai SY, Weigel NL, Edwards DP, Tsai MJ, O'Malley BW (1992a) Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. J Biol Chem 267(27):19513–19520

Allan GF, Tsai SY, Tsai MJ, O'Malley BW (1992b) Ligand-dependent conformational changes in the progesterone receptor are necessary for events that follow DNA binding. Proc Natl Acad Sci U S A 89(24):11750–11754

Amsterdam A, Tajima K, Sasson R (2002) Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochem Pharmacol 64(5–6):843–850

Aranda A, Pascual A (2001) Nuclear hormone receptors and gene expression. Physiol Rev 81(3):1269–1304

Bagchi MK, Elliston JF, Tsai SY, Edwards DP, Tsai MJ, O'Malley BW (1988) Steroid hormone-dependent interaction of human progesterone receptor with its target enhancer element. Mol Endocrinol 2(12):1221–1229. doi:10.1210/mend-2-12-1221

Bain DL, Franden MA, McManaman JL, Takimoto GS, Horwitz KB (2000) The N-terminal region of the human progesterone A-receptor. Structural analysis and the influence of the DNA binding domain. J Biol Chem 275(10):7313–7320

Bain DL, Franden MA, McManaman JL, Takimoto GS, Horwitz KB (2001) The N-terminal region of human progesterone B-receptors: biophysical and biochemical comparison to A-receptors. J Biol Chem 276(26):23825–23831. doi:10.1074/jbc.M102611200

Bain DL, Yang Q, Connaghan KD, Robblee JP, Miura MT, Degala GD, Lambert JR, Maluf NK (2012) Glucocorticoid receptor-DNA interactions: binding energetics are the primary determinant of sequence-specific transcriptional activity. J Mol Biol 422(1):18–32. doi:10.1016/j.jmb.2012.06.005

Barcroft J, Hill AV (1910) The nature of oxyhaemoglobin, with a note on its molecular weight. J Physiol 39(6):411–428

Baskakov IV, Kumar R, Srinivasan G, Ji YS, Bolen DW, Thompson EB (1999) Trimethylamine N-oxide-induced cooperative folding of an intrinsically unfolded transcription-activating fragment of human glucocorticoid receptor. J Biol Chem 274(16):10693–10696

Becker PB, Gloss B, Schmid W, Strahle U, Schutz G (1986) In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. Nature 324(6098):686–688. doi:10.1038/324686a0

Beekman JM, Allan GF, Tsai SY, Tsai MJ, O'Malley BW (1993) Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain. Mol Endocrinol 7(10):1266–1274. doi:10.1210/mend.7.10.8264659

Berglund H, Wolf-Watz M, Lundback T, van den Berg S, Hard T (1997) Structure and dynamics of the glucocorticoid receptor DNA-binding domain: comparison of wild type and a mutant with altered specificity. Biochemistry 36(37):11188–11197. doi:10.1021/bi970343i

Blackford JA, Jr., Guo C, Zhu R, Dougherty EJ, Chow CC, Simons SS, Jr. (2012) Identification of location and kinetically defined mechanism of cofactors and reporter genes in the cascade of steroid-regulated transactivation. J Biol Chem 287(49):40982–40995. doi:10.1074/jbc.M112.414805

Bolen DW, Baskakov IV (2001) The osmophobic effect: natural selection of a thermodynamic force in protein folding. J Mol Biol 310(5):955–963. doi:10.1006/jmbi.2001.4819

Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. Nature 375(6530):377–382. doi:10.1038/375377a0

Brodie J, McEwan IJ (2005) Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. J Mol Endocrinol 34(3):603–615. doi:10.1677/jme.1.01723

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389 (6652):753–758. doi:10.1038/39645

Buljan M, Chalancon G, Eustermann S, Wagner GP, Fuxreiter M, Bateman A, Babu MM (2012) Tissue-specific splicing of disordered segments that embed binding motifs rewires protein interaction networks. Mol Cell 46(6):871–883. doi:10.1016/j.molcel.2012.05.039

Cao Y, Bender IK, Konstantinidis AK, Shin SC, Jewell CM, Cidlowski JA, Schleimer RP, Lu NZ (2013) Glucocorticoid receptor translational isoforms underlie maturational stage-specific glucocorticoid sensitivities of dendritic cells in mice and humans. Blood 121(9):1553–1562. doi:10.1182/blood-2012-05-432336

Copik AJ, Webb MS, Miller AL, Wang Y, Kumar R, Thompson EB (2006) Activation function 1 of glucocorticoid receptor binds TATA-binding protein in vitro and in vivo. Mol Endocrinol 20(6):1218–1230. doi:10.1210/me.2005–0257

Dahlman-Wright K, Baumann H, McEwan IJ, Almlof T, Wright AP, Gustafsson JA, Hard T (1995) Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor. Proc Natl Acad Sci U S A 92(5):1699–1703

Daily MD, Gray JJ (2009) Allosteric communication occurs via networks of tertiary and quaternary motions in proteins. PLoS Comput Biol 5:e1000293

de Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, Brinkmann AO, White A, Brogan IJ, de Jong FH, Lamberts SW (2001) Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res 61(10):3937–3941

Dela Cruz FE, Kirsch DR, Heinrich JN (2000) Transcriptional activity of Drosophila melanogaster ecdysone receptor isoforms and ultraspiracle in Saccharomyces cerevisiae. J Mol Endocrinol 24(2):183–191

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC (2009) Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocr Rev 30(4):293–342. doi:10.1210/er.2009–0002

Dougherty JJ, Puri RK, Toft DO (1982) Phosphorylation in vivo of chicken oviduct progesterone receptor. J Biol Chem 257(23):14226–14230

Le Drean Y, Mincheneau N, Le Goff P, Michel D (2002) Potentiation of glucocorticoid receptor transcriptional activity by sumoylation. Endocrinology 143(9):3482–3489. doi:10.1210/en.2002–220135

Ferreon ACM, Ferreon JC, Wright PE, Deniz AA (2013) Modulation of allostery by protein intrinsic disorder. Nature 498:390–394

Fischer K, Kelly SM, Watt K, Price NC, McEwan IJ (2010) Conformation of the mineralocorticoid receptor N-terminal domain: evidence for induced and stable structure. Mol Endocrinol 24(10):1935–1948. doi:10.1210/me.2010–0005

Forman BM, Chen J, Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 94(9):4312–4317

Fu M, Wang C, Reutens AT, Wang J, Angeletti RH, Siconolfi-Baez L, Ogryzko V, Avantaggiati ML, Pestell RG (2000) p300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. J Biol Chem 275(27):20853–20860. doi:10.1074/jbc.M000660200

Garcia-Carpizo V, Ruiz-Llorente L, Fraga M, Aranda A (2011) The growing role of gene methylation on endocrine function. J Mol Endocrinol 47(2):R75–89. doi:10.1530/JME-11-0059

Garside H, Stevens A, Farrow S, Normand C, Houle B, Berry A, Maschera B, Ray D (2004) Glucocorticoid ligands specify different interactions with NF-kappaB by allosteric effects on the glucocorticoid receptor DNA binding domain. J Biol Chem 279(48):50050–50059. doi:10.1074/jbc.M407309200

Gee AC, Carlson KE, Martini PG, Katzenellenbogen BS, Katzenellenbogen JA (1999) Coactivator peptides have a differential stabilizing effect on the binding of estrogens and antiestrogens with the estrogen receptor. Mol Endocrinol 13(11):1912–1923. doi:10.1210/mend.13.11.0373

Gerber AN, Masuno K, Diamond MI (2009) Discovery of selective glucocorticoid receptor modulators by multiplexed reporter screening. Proc Natl Acad Sci U S A 106(12):4929–4934. doi:10.1073/pnas.0812308106

Geserick C, Meyer HA, Barbulescu K, Haendler B (2003) Differential modulation of androgen receptor action by deoxyribonucleic acid response elements. Mol Endocrinol 17(9):1738–1750. doi:10.1210/me.2002–0379

Glass CK, Holloway JM, Devary OV, Rosenfeld MG (1988) The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. Cell 54(3):313–323

Greenfield N, Vijayanathan V, Thomas TJ, Gallo MA, Thomas T (2001) Increase in the stability and helical content of estrogen receptor alpha in the presence of the estrogen response element: analysis by circular dichroism spectroscopy. Biochemistry 40(22):6646–6652

Hake SB, Garcia BA, Duncan EM, Kauer M, Dellaire G, Shabanowitz J, Bazett-Jones DP, Allis CD, Hunt DF (2006) Expression patterns and post-translational modifications associated with mammalian histone H3 variants. J Biol Chem 281(1):559–568. doi:10.1074/jbc.M509266200

Han SJ, DeMayo FJ, Xu J, Tsai SY, Tsai MJ, O'Malley BW (2006) Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. Mol Endocrinol 20(1):45–55. doi:10.1210/me.2005–0310

Henry ER, Bettati S, Hofrichter J, Eaton WA (2002) A tertiary two-state allosteric model for hemoglobin. Biophys Chem 98(1–2):149–164

Hilser VJ, Thompson EB (2007) Intrinsic disorder as a mechanism to optimize allosteric coupling in proteins. Proc Natl Acad Sci U S A 104(20):8311–8315. doi:10.1073/pnas.0700329104

Hilser VJ, Wrabl JO, Motlagh HN (2012) Structural and energetic basis of allostery. Annu Rev Biophys 41:585–609

Housley PR, Pratt WB (1983) Direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. J Biol Chem 258(7):4630–4635

Hsu PY, Hsu HK, Singer GA, Yan PS, Rodriguez BA, Liu JC, Weng YI, Deatherage DE, Chen Z, Pereira JS, Lopez R, Russo J, Wang Q, Lamartiniere CA, Nephew KP, Huang TH (2010) Estrogen-mediated epigenetic repression of large chromosomal regions through DNA looping. Genome Res 20(6):733–744. doi:10.1101/gr.101923.109

Hudson WH, Youn C, Ortlund EA (2013) The structural basis of direct glucocorticoid-mediated transrepression. Nat Struct Mol Biol 20(1):53–58. doi:10.1038/nsmb.2456

Ikeda M, Wilcox EC, Chin WW (1996) Different DNA elements can modulate the conformation of thyroid hormone receptor heterodimer and its transcriptional activity. J Biol Chem 271(38):23096–23104

Ivarie RD, O'Farrell PH (1978) The glucocorticoid domain: steroid-mediated changes in the rate of synthesis of rat hepatoma proteins. Cell 13(1):41–55

Jenkins BD, Pullen CB, Darimont BD (2001) Novel glucocorticoid receptor coactivator effector mechanisms. Trends Endocrinol Metab 12(3):122–126

Kallenberger BC, Love JD, Chatterjee VK, Schwabe JW (2003) A dynamic mechanism of nuclear receptor activation and its perturbation in a human disease. Nat Struct Biol 10(2):136–140. doi:10.1038/nsb892

Kangaspeska S, Stride B, Metivier R, Polycarpou-Schwarz M, Ibberson D, Carmouche RP, Benes V, Gannon F, Reid G (2008) Transient cyclical methylation of promoter DNA. Nature 452(7183):112–115. doi:10.1038/nature06640

Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. The EMBO journal 9(5):1603–1614

Khan SH, Awasthi S, Guo C, Goswami D, Ling J, Griffin PR, Simons SS, Jr., Kumar R (2012) Binding of the N-terminal region of coactivator TIF2 to the intrinsically disordered AF1 domain of the glucocorticoid receptor is accompanied by conformational reorganizations. J Biol Chem 287(53):44546–44560. doi:10.1074/jbc.M112.411330

King WJ, Greene GL (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307(5953):745–747
Knutti D, Kaul A, Kralli A (2000) A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. Mol Cell Biol 20(7):2411–2422
Koshland DE, Jr., Nemethy G, Filmer D (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 5(1):365–385
Krasowski MD, Reschly EJ, Ekins S (2008) Intrinsic disorder in nuclear hormone receptors. J Proteome Res 7(10):4359–4372. doi:10.1021/pr8003024
Kumar R, Thompson EB (2010) Influence of flanking sequences on signaling between the activation function AF1 and DNA-binding domain of the glucocorticoid receptor. Arch Biochem Biophys 496(2):140–145. doi:10.1016/j.abb.2010.02.010
Kumar R, Baskakov IV, Srinivasan G, Bolen DW, Lee JC, Thompson EB (1999) Interdomain signaling in a two-domain fragment of the human glucocorticoid receptor. J Biol Chem 274(35):24737–24741
Kumar R, Lee JC, Bolen DW, Thompson EB (2001) The conformation of the glucocorticoid receptor af1/tau1 domain induced by osmolyte binds co-regulatory proteins. J Biol Chem 276(21):18146–18152. doi:10.1074/jbc.M100825200
Kumar R, Volk DE, Li J, Lee JC, Gorenstein DG, Thompson EB (2004) TATA box binding protein induces structure in the recombinant glucocorticoid receptor AF1 domain. Proc Natl Acad Sci U S A 101(47):16425–16430. doi:10.1073/pnas.0407160101
Lavery DN, McEwan IJ (2005) Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem J 391(Pt 3):449–464. doi:10.1042/BJ20050872
Lavery DN, McEwan IJ (2008) Structural characterization of the native NH2-terminal transactivation domain of the human androgen receptor: a collapsed disordered conformation underlies structural plasticity and protein-induced folding. Biochemistry 47(11):3360–3369. doi:10.1021/bi702221e
Lefstin JA, Thomas JR, Yamamoto KR (1994) Influence of a steroid receptor DNA-binding domain on transcriptional regulatory functions. Gen Dev 8(23):2842–2856
Li X, Zhang S, Blander G, Tse JG, Krieger M, Guarente L (2007) SIRT1 deacetylates and positively regulates the nuclear receptor LXR. Mol Cell 28(1):91–106. doi:10.1016/j.molcel.2007.07.032
Li J, Motlagh HN, Chakuroff C, Thompson EB, Hilser VJ (2012) Thermodynamic dissection of the intrinsically disordered N-terminal domain of human glucocorticoid receptor. J Biol Chem 287(32):26777–26787. doi:10.1074/jbc.M112.355651
Liu J, Perumal NB, Oldfield CJ, Su EW, Uversky VU, Dunker AK (2006) Intrinsic disorder in transcription factors. Biochemistry 45:6873–6888
Lockless SW, Ranganathan R (1999) Evolutionarily conserved pathways of energetic connectivity in protein families. Science 286:295–299
Loven MA, Wood JR, Nardulli AM (2001) Interaction of estrogen receptors alpha and beta with estrogen response elements. Mol Cell Endocrinol 181(1–2):151–163
Lu NZ, Cidlowski JA (2005) Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell 18(3):331–342. doi:10.1016/j.molcel.2005.03.025
Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352(6335):497–505. doi:10.1038/352497a0
M.F. P (1970) Stereochemistry of cooperative effects in haemoglobin. Nature 228:726–734
Martinez E, Wahli W (1989) Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. The EMBO J 8(12):3781–3791
Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324(5925):407–410. doi:10.1126/science.1164265
Meyer ME, Pornon A, Ji JW, Bocquel MT, Chambon P, Gronemeyer H (1990) Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. The EMBO J 9(12):3923–3932

Monod J, Wyman J, Changeux JP (1965) On the nature of allosteric transitions: a plausible model. J Mol Biol 12:88–118

Motlagh HN, Hilser VJ (2012) Agonism/antagonism switching in allosteric ensembles. Proc Natl Acad Sci U S A 109(11):4134–4139. doi:10.1073/pnas.1120519109

Motlagh HN, Li J, Thompson EB, Hilser VJ (2012) Interplay between allostery and intrinsic disorder in an ensemble. Biochem Soc Trans 40(5):975

Mouillet JF, Henrich VC, Lezzi M, Vogtli M (2001) Differential control of gene activity by isoforms A, B1 and B2 of the Drosophila ecdysone receptor. Eur J Biochem 268(6):1811–1819

Nahoum V, Perez E, Germain P, Rodriguez-Barrios F, Manzo F, Kammerer S, Lemaire G, Hirsch O, Royer CA, Gronemeyer H, de Lera AR, Bourguet W (2007) Modulators of the structural dynamics of the retinoid X receptor to reveal receptor function. Proc Natl Acad Sci U S A 104(44):17323–17328. doi:10.1073/pnas.0705356104

Nie Z, Xue Y, Yang D, Zhou S, Deroo BJ, Archer TK, Wang W (2000) A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol Cell Biol 20(23):8879–8888

Nocula-Ługowska M, Rymarczyk G, Lisowski M, Ożyhar A (2009) Isoform-specific variation in the intrinsic disorder of the ecdysteroid receptor N-terminal domain. Proteins 76(2):291–308. doi:10.1002/prot.22342

Norman AW, Mizwicki MT, Norman DP (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov 3(1):27–41. doi:10.1038/nrd1283

Perutz MF, Fermi G, Luisi B, Shaanan B, Liddington RC (1987) Stereochemistry of cooperative mechanisms in hemoglobin. Cold Spring Harb Symp Quant Biol 52:555–565

Perutz MF, Wilkinson AJ, Paoli M, Dodson GG (1998) The stereochemical mechanism of the cooperative effects in hemoglobin revisited. Annu Rev Biophys Biomol Struct 27:1–34

Petit CM, Zhang J, Sapienza PJ, Fuentes EJ, Lee AL (2009) Hidden dynamic allostery in a PDZ domain. Proc Natl Acad Sci U S A 106(43):18249–18254

Pike JW, Sleator NM (1985) Hormone-dependent phosphorylation of the 1,25-dihydroxyvitamin D3 receptor in mouse fibroblasts. BiochemBiophys Res Commun 131(1):378–385

Poukka H, Karvonen U, Janne OA, Palvimo JJ (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci U S A 97(26):14145–14150. doi:10.1073/pnas.97.26.14145

Reichheld SE, Yu Z, Davidson AR (2009) The induction of folding cooperativity by ligand binding drives the allosteric response of tetracycline repressor. Proc Natl Acad Sci 106(52):22263–22268. doi:10.1073/pnas.0911566106

Reid J, Kelly SM, Watt K, Price NC, McEwan IJ (2002) Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions. J Biol Chem 277(22):20079–20086. doi:10.1074/jbc.M201003200

Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. Nature 378(6558):681–689. doi:10.1038/378681a0

Ronacher K, Hadley K, Avenant C, Stubsrud E, Simons SS, Jr., Louw A, Hapgood JP (2009) Ligand-selective transactivation and transrepression via the glucocorticoid receptor: role of cofactor interaction. Mol Cell Endocrinol 299(2):219–231. doi:10.1016/j.mce.2008.10.008

Russcher H, van Rossum EF, de Jong FH, Brinkmann AO, Lamberts SW, Koper JW (2005) Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. Mol Endocrinol 19(7):1687–1696. doi:10.1210/me.2004–0467

Russcher H, Dalm VA, de Jong FH, Brinkmann AO, Hofland LJ, Lamberts SW, Koper JW (2007) Associations between promoter usage and alternative splicing of the glucocorticoid receptor gene. J Mol Endocrinol 38(1–2):91–98. doi:10.1677/jme.1.02117

Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson JA, Rottman FM, Yamamoto KR (1988) Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Gen Dev 2(9):1144–1154

Schrader WT, O'Malley BW (1972) Progesterone-binding components of chick oviduct. IV. Characterization of purified subunits. J Biol Chem 247(1):51–59
Schwabe JW, Neuhaus D, Rhodes D (1990) Solution structure of the DNA-binding domain of the oestrogen receptor. Nature 348(6300):458–461. doi:10.1038/348458a0
Schwabe JW, Chapman L, Rhodes D (1995) The oestrogen receptor recognizes an imperfectly palindromic response element through an alternative side-chain conformation. Structure 3(2):201–213
Shang Y, Brown M (2002) Molecular determinants for the tissue specificity of SERMs. Science 295(5564):2465–2468. doi:10.1126/science.1068537
Smith FR, Ackers GK (1985) Experimental resolution of cooperative free energies for the ten ligation states of human hemoglobin. Proc Natl Acad Sci U S A 82:5347–5351
Spolar RS, Record MT, Jr. (1994) Coupling of local folding to site-specific binding of proteins to DNA. Science 263(5148):777–784
Starr DB, Matsui W, Thomas JR, Yamamoto KR (1996) Intracellular receptors use a common mechanism to interpret signaling information at response elements. Gen Dev 10(10):1271–1283
Suel GM, Lockless SW, Wall MA, Ranganathan R (2003) Evolutionarily conserved networks of residues mediate allosteric communication in proteins. Nat Struct Biol 10:59–69
Szapary D, Xu M, Simons SS, Jr. (1996) Induction properties of a transiently transfected glucocorticoid-responsive gene vary with glucocorticoid receptor concentration. J Biol Chem 271(48):30576–30582
Szapary D, Huang Y, Simons SS, Jr. (1999) Opposing effects of corepressor and coactivators in determining the dose-response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated gene expression. Mol Endocrinol 13(12):2108–2121. doi:10.1210/mend.13.12.0384
Talbot WS, Swyryd EA, Hogness DS (1993) Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73(7):1323–1337
Thompson EB, Kumar R (2003) DNA binding of nuclear hormone receptors influences their structure and function. Biochem Biophys Res Commun 306(1):1–4
Tompa P (2011) Unstrucural biology coming of age. Curr Opin Struct Biol 21:409–425
Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM (1994) mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Gen Dev 8(10):1224–1234
Truman JW, Talbot WS, Fahrbach SE, Hogness DS (1994) Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development. Development 120(1):219–234
Uversky VN, Oldfield CJ, Dunker AK (2005) Showing your ID: intrinsic disorder as an ID for recognition, regulation and cell signaling. J Mol Recognit: JMR 18(5):343–384. doi:10.1002/jmr.747
Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ (1995) A structural role for hormone in the thyroid hormone receptor. Nature 378(6558):690–697. doi:10.1038/378690a0
Wallace AD, Cidlowski JA (2001) Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J Biol Chem 276(46):42714–42721. doi:10.1074/jbc.M106033200
Walters KA, Simanainen U, Handelsman DJ (2010) Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. Hum Reprod Update 16(5):543–558. doi:10.1093/humupd/dmq003
Warnmark A, Wikstrom A, Wright AP, Gustafsson JA, Hard T (2001) The N-terminal regions of estrogen receptor alpha and beta are unstructured in vitro and show different TBP binding properties. J Biol Chem 276(49):45939–45944. doi:10.1074/jbc.M107875200
Watters AL, Deka P, Corrent C, Callender D, Varani G, Sosnick T, Baker D (2007) The highly cooperative folding of small naturally occurring proteins is likely the result of natural selection. Cell 128(3):613–624. doi:10.1016/j.cell.2006.12.042
Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M, Cidlowski JA (1997) Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. J Biol Chem 272(14):9287–9293

Welshons WV, Lieberman ME, Gorski J (1984) Nuclear localization of unoccupied oestrogen receptors. Nature 307(5953):747–749

Wolf DM, Jordan VC (1992) Gynecologic complications associated with long-term adjuvant tamoxifen therapy for breast cancer. Gynecol Oncol 45(2):118–128

Wood JR, Greene GL, Nardulli AM (1998) Estrogen response elements function as allosteric modulators of estrogen receptor conformation. Mol Cell Biol 18(4):1927–1934

Wood JR, Likhite VS, Loven MA, Nardulli AM (2001) Allosteric modulation of estrogen receptor conformation by different estrogen response elements. Mol Endocrinol 15(7):1114–1126. doi:10.1210/mend.15.7.0671

Wyman J, Gill SJ (1990) Binding and Linkage: functional chemistry of biological macromolecules. Mill Valley, CA: University Science Books. ISBN 0-935702-56-3

Yamamoto KR, Alberts BM (1972) In vitro conversion of estradiol-receptor protein to its nuclear form: dependence on hormone and DNA. Proc Natl Acad Sci U S A 69(8):2105–2109

Structural Analyses of Ordered and Disordered Regions in Ecdysteroid Receptor

Marek Orłowski, Piotr Dobryszycki, Anna Zoglowek, Joanna Pieprzyk, Katarzyna Bielska, Beata Greb-Markiewicz, Małgorzata Kozłowska, Aneta Tarczewska and Andrzej Ożyhar

1 Introduction

Insect development, reproduction and other important biological processes are regulated by two classes of lipid-soluble hormones, ecdysteroids and juvenile hormones (JHs). The multivalent actions of the ecdysteroid are mediated at the molecular level by a heterodimeric receptor complex comprised of the ecdysteroid receptor (EcR) and the ultraspiracle (Usp) (Yao et al. 1992, 1993). Both proteins are members of the nuclear receptor superfamily and similarly to other nuclear receptors (NRs) EcR and Usp share a common structural organization and exhibit a highly modular structure. In particular these proteins are made up of both, stably folded globular domains involved in ligand and DNA binding (LBD, DBD, respectively) and intrinsically disordered regions (IDRs), including a highly variable A/B region called the N-terminal domain (NTD) and a hinge region linking LBD and DBD. Some EcRs and Usps contain an additional, non-conserved C-terminal region, called F-domain (McEwan 2009).

2 Structural Analysis of the DBD and LBD

2.1 Structural Plasticity of the Ecdysteroid Receptor Complex on the Natural Response Element

EcR/Usp heterodimers recognize the ecdysteroid response elements (EcREs) present in the promoter regions of the 20-hydroxyecdysone (20E) response genes. Most naturally occurring EcREs are quasi-palindromes with a single nucleotide spacer

A. Ożyhar (✉) · M. Orłowski · P. Dobryszycki · A. Zoglowek · J. Pieprzyk · K. Bielska · B. Greb-Markiewicz · M. Kozłowska · A. Tarczewska
Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
e-mail: andrzej.ozyhar@pwr.edu.pl

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_6

(Riddihough and Pelham 1986, 1987; Cherbas et al. 1991; Antoniewski et al. 1994, 1996; Lehmann and Korge 1995; Lehmann et al. 1997), as opposed to the 3-bp spacer that is typical of vertebrate steroid response elements (Aagaard et al. 2011). Additionally, the EcR/Usp heterocomplex also recognizes directly-repeated elements (D'Avino et al. 1995; Antoniewski et al. 1996; Wang et al. 1998). The first natural EcRE was identified in the promoter of the *Drosophila melanogaster* heat-shock protein-27 gene ($hsp27_{pal}$) (Riddihough and Pelham 1986), and it is still the best described and characterized quasi-palindromic EcRE. Palindromic hexameric repeats impose a symmetrical structure that results in a head-to-head arrangement of the DBDs with each DBD of the vertebrate steroid receptor homodimer analogously making contact with one half-site (Aagaard et al. 2011). However, in contrast to vertebrate palindromic elements, palindromic EcREs are very degenerate and can be regarded as highly asymmetric response elements with the ability to dictate the orientation of heterodimeric complexes on the proper promoter (Riddihough and Pelham 1986; Ożyhar et al. 1991; Ożyhar and Pongs 1993; Niedziela-Majka et al. 2000). Electrophoretic mobility shift assays revealed that $hsp27_{pal}$ half-sites contribute in different ways to the binding of the EcR/Usp heterocomplex (Niedziela-Majka et al. 2000). In particular, the 5'half-site exhibits higher affinity for both DBDs than the 3'half-site. Moreover, there is stronger binding of the UspDBD to the 5'half-site than with the EcRDBD. The data showed that UspDBD may serve as an anchor locating the heterocomplex in a definite orientation (5'-UspDBD/EcRDBD-3') on the $hsp27_{pal}$ (Niedziela-Majka et al. 2000). Interestingly, in the absence of the UspDBD, the EcRDBD molecule is able to bind $hsp27_{pal}$ as homodimers (Niedziela-Majka et al. 2000). On the other hand, full-length EcR exhibits virtually no tendency to form homodimers when binding to asymmetric or symmetric elements, as was shown by Perera *et al.* (Perera et al. 2005). One explanation for such a range of activity is that EcR and Usp utilize different dimerization interfaces for binding to symmetric and asymmetric response elements (Perera et al. 2005). The unique characteristic of the EcRDBD molecule is its plasticity and adaptability (Orłowski et al. 2004). The data indicated that there was remarkable tolerance with respect to mutations involving the DNA-binding function of the EcRDBD and during the formation of a complex with the UspDBD on the $hsp27_{pal}$. Circular dichroism (CD) spectra analyses and protein unfolding experiments indicated a lower α-helix content for the EcRDBD in comparison to the UspDBD and less stability in solution (Orłowski et al. 2004). The dissymmetry observed in the molecular properties of the UspDBD and EcRDBD may be a key factor that allows the EcR/Usp heterocomplex to mediate crucial events in the ecdysteroid signaling pathways, by exploiting different DNA-binding modes to recognize and bind the cognate response element.

A high degree of structural plasticity enables EcR to form complexes not only with Usp but also with other NRs. In particular, EcR is able to interact with the *Drosophila* hormone receptor 38 (DHR38/NR4A4) (Zoglowek et al. 2012), the ortholog of the mammalian NGFI-B subfamily of orphan NR, which includes NGFI-B (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3) (Fisk and Thummel 1995; Sutherland et al. 1995; Laudet 2002). Three independent methods: gel retardation analy-

sis, a DNaseI footprinting assay, and fluorescence anisotropy measurements—have demonstrated that the EcRDBD is able to interact with the DHR38DBD on *hsp27*$_{pal}$ (Zoglowek et al. 2012). However, interaction between EcR and DHR38 is not restricted to their DBDs. Full-length EcR and DHR38 can form specific complexes within the nuclei of living cells (Zoglowek et al. 2012). *In vitro* experiments showed that full-length DHR38 can also form stable complexes with Usp and thus compete with EcR for heterodimerization and consequently, disrupt the binding of Usp and EcR to the *hsp27*$_{pal}$ response element (Sutherland et al. 1995). The biological and physiological significance of the interaction between EcR and DHR38 remains unknown.

The molecular basis of the conformational instability and flexibility of the *Drosophila* EcRDBD was recently elucidated (Szamborska-Gbur et al. 2014). Aided by the use of an *in silico* protein design, the biochemical data on the mutants indicated that non-conserved residues within the second α-helix from the second Zn^{2+}-module, which are involved in the formation of the EcRDBD hydrophobic core, are specific structural elements that may contribute to the instability of the EcRDBD (Szamborska-Gbur et al. 2014). L58 is likely to be the key residue that was responsible for the low stability of the EcRDBD that was observed earlier by Orłowski *et al.* (Orłowski et al. 2004).

The structures of the NRs, DBDs and LBDs and their functional allosteric correlations have been investigated in numerous studies and extensively described in other reviews. There were four studies on the structure of the EcRDBD and UspDBD in insects using biochemical, biophysical and crystallographic research (Devarakonda et al. 2003; Jakób et al. 2007; Orłowski et al. 2004; Szamborska-Gbur et al. 2014). Two crystal structures were found comprising an idealized IR-1 element (Devarakonda et al. 2003) and the natural *hsp27*$_{pal}$ sequence (Jakób et al. 2007), providing detailed insight into the UspDBD/EcRDBD structure bound to DNA (Fig. 1). Based on the data mentioned above (Niedziela-Majka et al. 2000; Devarakonda et al. 2003), the heterodimer interacts with the *hsp27*$_{pal}$ element with a defined polarity, where the UspDBD is bound to the 5'half-site and the EcRDBD to the 3'half-site of *hsp27*$_{pal}$ (Niedziela-Majka et al. 2000; Jakób et al. 2007). The overall polarity was similar to that observed for the retinoid X receptor (RXR) heterodimers bound with asymmetric elements (Rastinejad 2001). The structure of UspDBD/EcRDBD-*hsp27*$_{pal}$ was significantly different in comparison with the UspDBD/EcRDBD-IR1 structure. Although the overall fold of these two heterodimers is similar, the structure of the UspDBD/EcRDBD heterocomplex interacting with the natural sequence revealed a total of eleven additional amino acid residues and some of them are involved in DNA-binding : three at the N-terminus of the EcRDBD (R-6, V-5, Q-4), six in the C-terminal extension (CTE) of the EcRDBD (Q76, C77, A78, M79, K80, R81), i.e. within the N-terminal part corresponding to the A-box (Niedziela-Majka et al. 2000), and one residue at each end of the UspDBD (K3, R75) (Jakób et al. 2007). An extraordinary feature of the UspDBD/EcRDBD-*hsp27*$_{pal}$ complex is an α-helix clearly visible in the CTE of the EcRDBD (Fig. 1). The CTE, including the T-box and A-box, of the EcRDBD was shown to be a crucial element involved in DNA-binding (Niedziela-Majka et al. 2000; Orłowski

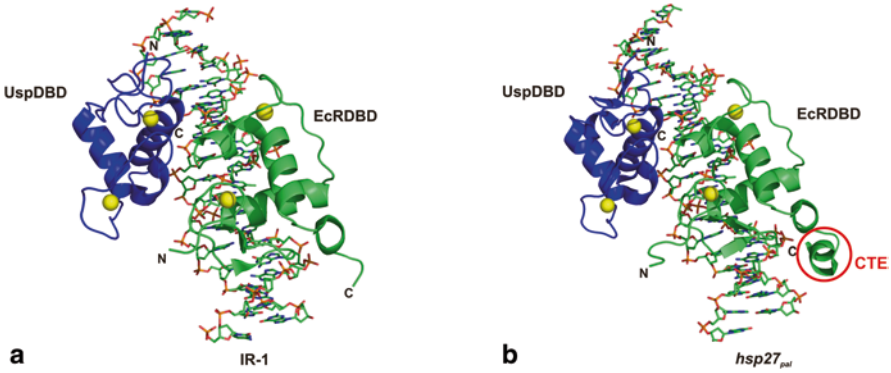


Fig. 1 The UspDBD/EcRDBD heterocomplex bound to an idealized consensus IR-1 (Devarakonda et al. 2003) (**a**) and to the natural *hsp27*$_{pal}$ (Jakób et al. 2007) (**b**). The structure of the UspDBD/EcRDBD heterocomplex formed with the natural quasi-palindromic EcRE (*hsp27*$_{pal}$) (**b**) exhibits significant differences in comparison to the UspDBD/EcRDBD-IR1 structure (**a**). A comparative analysis clearly showed how the EcRDBD accommodates DNA-induced structural changes. In particular, a fragment of the C-terminal extension (CTE) (*red circle*) of the EcRDBD folded into an α-helix, which was not seen in the structure obtained with the IR-1 element. The structure of the UspDBD/EcRDBD-*hsp27*$_{pal}$ (Jakób et al. 2007) confirms the biochemical studies revealing the importance of key residues and segments from the UspDBD and EcRDBD molecules that are involved in the specific recognition of the natural *hsp27*$_{pal}$ (Niedziela-Majka et al. 2000; Grad et al. 2001; Orłowski et al. 2004). The UspDBD/EcRDBD-IR-1 structure is based on the file deposited in PDB (accession code: 1R0O) (**a**) and the UspDBD/EcRDBD-*hsp27*$_{pal}$ heterocomplex structure is based on the file deposited in PDB (accession code: 2HAN) (**b**). The structures were visualized with PyMOL 0.99 (Delano 2002)

et al. 2004). In contrast to the high sequence conservation of the core DBD region within the NRs, the CTE sequences are not well conserved. The CTE was suggested to play an important role in the ability of the DBD to discriminate the response element interacting as homo- and heterodimers with asymmetrical, directly repeated elements (Rastinejad 2001). Furthermore, the structure of the UspDBD/EcRDBD heterocomplex on the natural *hsp27*$_{pal}$ revealed that the α-helix from the CTE of the EcRDBD that is positioned within the minor groove of the *hsp27*$_{pal}$ does not match any of the locations observed in the NR DBD structures (Jakób et al. 2007). A comparative and detailed analysis of two crystal structures of the UspDBD/EcRDBD heterocomplex clearly shows how the flexible EcRDBD accommodates the DNA-induced structural changes. Recently, the cryo-electron microscopy structure of the *Heliothis virescens* Usp/EcR complex bound to the *hsp27* element was elucidated for the N-terminally truncated Usp and EcR, i.e. lacking the highly variable and intrinsically disordered A/B domains (Maletta et al. 2014). The structure revealed that the overall architecture of the DNA-bound heterodimer is asymmetric, despite the quasi-symmetric nature of the *hsp27* element (Maletta et al. 2014) exactly as it was suggested for the first time by Niedziela *et al.* (Niedziela-Majka et al. 2000). The most striking feature of the *H. virescens* Usp/EcR-*hsp27* complex is that the

LBD of Usp, lacking its NTD, is in close proximity to the 5' flanking sequence of the DNA used in the study. The basic residues from the helix H9 of Usp are suggested to be important in functional interactions that help fine-tuning transcriptional activity by modulating the ecdysteroid-binding properties of the ecdysone receptor (Maletta et al. 2014).

The intrinsic plasticity and adaptability of the EcRDBD molecule may be a key element which enables the protein to recognize a broad range of EcREs. Although there have been in-depth studies on the structure and function of the ecdysteroid receptor in arthropods, the high-resolution structure of the full-length EcR/Usp heterodimer alone, in complex with a specific EcRE, or with partners crucial to 20E and JH action, is still waiting to be resolved.

2.2 Conformational Changes of the Ecdysteroid Receptor on the Natural Response Element-Fluorescence Studies

2.2.1 DNA Binding Domain Dynamics and Detecting the Bending of the hsp27 Response Element

Many years ago, transcription factors, including NRs, were shown to induce substantial distortions in DNA structure, which may have affected the transcription-inducing activity of other complexes (Shulemovich et al. 1995; Lane et al. 1992; Potthoff et al. 1996; Prendergast et al. 1996; Petz et al. 1997; Heyduk et al. 1997). As described above, the UspDBD preferentially binds the 5'half-site of $hsp27_{pal}$ and acts as an anchor that dictates the polarity of the EcR/Usp heterocomplex (5'-Usp-DBD-EcR-DBD-3') (Niedziela-Majka et al. 2000). The final structure depends on both protein conformation and a regulatory element. The UspDBD alone and the UspDBD/EcRDBD heterodimer induce $hsp27_{pal}$ bending, however, the UspDBD plays a crucial role in defining the overall architecture of the UspDBD/EcRDBD-$hsp27_{pal}$ complex (Niedziela-Majka et al. 2000; Dobryszycki et al. 2006). In contrast to the UspDBD, the EcRDBD is flexible and it causes a slight additional conformational change in the preformed structure (Dobryszycki et al. 2006; Orłowski et al. 2004). Nevertheless, FRET analysis (fluorescence resonance energy transfer) indicated that both proteins are distorted, which changes the distances between the respective protein ends and the resulting DNA ends (Fig. 2). These spatial relationships indicate that when the UspDBD bound to the regulatory element 5'half-site there is a reduction in the distance between the ends of $hsp27_{pal}$ and bending of about 23° (Dobryszycki et al. 2006; Jakób et al. 2007). The binding of UspDBD brings both ends of the DNA to the C-terminus of the UspDBD. This may be the result of DNA bending, but it is also most likely the result of a conformational change in the protein (Fig. 2a). The binding of the EcRDBD to the DNA 3'half-site, in the presence of the UspDBD at the 5'half-site, does not significantly alter the DNA bending angle, but it clearly causes the C-end of the UspDBD to be about 6Å from both the 5'- and 3'-ends of $hsp27_{pal}$. This indicates that there is a strong conformational

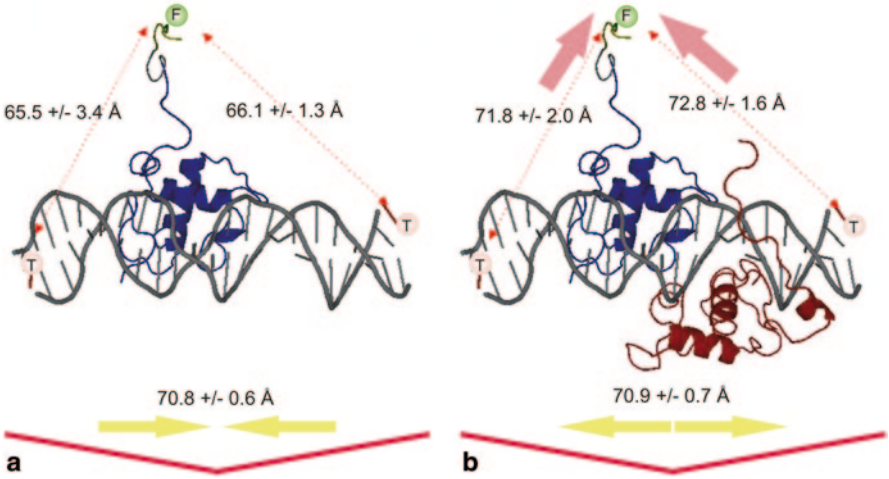


Fig. 2 Changes in the distances in the complexes between the UspDBD (*blue ribbon*) and the $hsp27_{pal}$ regulatory element in the absence **a** and in the presence **b** of the EcRDBD (*brown ribbon*). The figure was prepared on the basis of the structure of the complex of the ecdysteroid receptor DBDs with $hsp27_{pal}$ obtained from X-ray experiments (Jakób et al. 2007). Taken from Pakuła et al. (2012) (Pakuła et al. 2012)

change in the UspDBD (Fig. 2b). The changes in the distances could have come from structural distortions in the protein or the regulatory element. An open question remains concerning DNA hinge point(s) and the best model to use to describe bending. It is important to note that the FRET results were in full agreement with the X-ray structure.

DHR38 is the *Drosophila* ortholog of the mammalian NGFI-B subfamily of orphan NR, which includes NGFI-B (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3). Although members of the NGFI-B family are thought to function exclusively as monomers, DHR38 in fact interacts strongly with Usp, and this interaction is evolutionarily conserved (Sutherland et al. 1995; Dutko-Gwóźdź et al. 2008). DHR38 can repress transcription of a reporter gene under the control of the $hsp27_{pal}$. *In vitro* experiments have shown that full-length DHR38 can form stable complexes with Usp, and thus can compete with EcR as a dimerization partner for Usp, which consequently disrupts the binding of EcR/Usp to the response element (Sutherland et al. 1995). Surprisingly, recent data indicated that the EcRDBD is able to interact with the DHR38DBD on the $hsp27_{pal}$ (Zoglowek et al. 2012). Based on the end-to-end distances between donor and acceptor probes, $hsp27_{pal}$ bend angles were estimated using FRET method (Dobryszycki et al. 2006). The results obtained from FRET measurements revealed that there had been shortening of the end-to-end distance of $hsp27_{pal,}$ both in the presence of the EcRDBD and also in the presence of the EcRDBD and DHR38DBD together. This corresponds to the respective DNA bend angles of about 36.2° and 33.6°, respectively. The small 3.4° decrease in the bend angle in the presence of both proteins suggests that the key factor involved in

the bending is the binding of the EcRDBD molecule to the 5'-regulatory element half-site. It seems that the EcRDBD induced a conformational change in $hsp27_{pal}$ and created the conditions needed for DHR38DBD binding. It is worth noting that the interaction of the EcRDBD induced bending in $hsp27_{pal}$, while subsequent binding of the DHR38DBD did not significantly change the overall distortion of the regulatory element.

2.2.2 Cooperation Within the Protein-Protein Interaction Between the UspDBD and EcRDBD

Quantification of the level of DNA-binding activity of specific proteins is one of the most commonly performed experiments in biomedical research. Molecular beacons (MBs) use FRET measurements to investigate ssDNA hybridization and protein–DNA interactions in solution (Dummitt and Chang 2006; Li 2000; Fang et al. 2000; Tyagi and Kramer 1996; Heyduk et al. 2003; Heyduk and Heyduk 2002; Knoll and Heyduk 2004; Krusiński et al. 2008). A technology based on MBs also makes it possible to quantitatively investigate proteins that interact with DNA (Krusiński et al. 2010). The double molecular beacon (DMBs) system has been developed for independent quantitative analysis of the binding affinity of dimerizing DNA-binding proteins with two specific DNA sites in proximity to each other. The use of DMBs showed that EcRDBD/UspDBD interactions mediate the cooperative binding of the ecdysteroid receptor DBDs to $hsp27_{pal}$. An analysis of the microscopic dissociation constants obtained with a DMB led to the conclusion that there is increased affinity of the UspDBD to the 5'half-site in the presence of the EcRDBD when the 3'half-site was occupied, and there was increased affinity of the EcRDBD to the 3'half-site when the 5'half-site was occupied. This cooperative effect was quantified by the respective dissociation constants, clearly indicating that protein-DNA interactions are strongly influenced by UspDBD/EcRDBD interactions.

2.3 The EcRLBD/UspLBD Heterocomplex

To date, five crystallographic structures have been described for the LBDs of EcR/Usp heterodimers with bound ligands. The reported structures are from moth *Heliothis virescens* (*Lepidoptera*) (Browning et al. 2007) the whitefly *Bemisia tabaci* (*Hemiptera*) (Carmichael et al. 2005) and the beetle *Tribolium castaneum* (*Coleoptera*) (Iwema et al. 2007) in complex with phyto-ecdysteroid ponasterone A (ponA), which differs from 20E only in the lack of the 25-hydroxyl group. LBDs from *Heliothis* was also reported in complex with 20E (Billas et al. 2003). There is also an additional structure that was reported from *Heliothis* EcRLBD/UspLBD in complex with a non-steroidal, lepidopteran-specific agonist BYI06830 dibenzoylhydrazine (DBH insecticide) (Billas et al. 2003). The overall architecture of the EcRLBD and UspLBD that makes up each heterodimer is similar to that seen

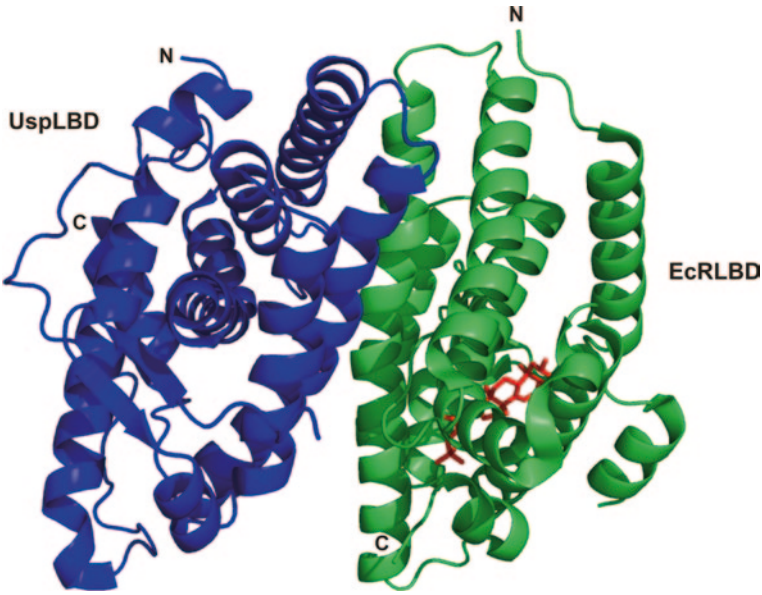


Fig. 3 The overall structure of the *Heliothis* EcRLBD/UspLBD bound to its endogenous ligand 20E (*red*) with EcR in *green* and USP in *blue* (Browning et al. 2007). The heterodimer structure is based on the file deposited in PDB (accession code: 2R40). The structure was visualized with PyMOL 0.99 (Delano 2002)

in the crystal structures of other NR LBDs, with a general fold consisting of a three-layered, antiparallel, α-helical sandwich and a β-sheet. The ligand-binding pocket has a J-shaped architecture that extends from helix H12 to helix H5 and the β-sheet, and it is completely buried inside the receptor. The overall structure of the *Heliothis* heterodimer EcRLBD/UspLBD bound to 20E (Fig. 3) is highly homologous to the structure of the ponA bound *Heliothis*, *Bemisia*, and *Tribolium* receptor complexes; the interactions between the residues of the ligand-binding pocket and 20E or ponA are highly conserved (Browning et al. 2007). In their respective ligand bound EcRLBD/UspLBD heterodimer crystal structures, 20E and ponA adopt almost identical chair conformations and have similar positioning of their alkyl tail (Browning et al. 2007). The ligand/receptor interactions are similar, except for the extra hydrogen bond created between the 25-OH group of 20E and the polar residue Asn504 in helix H11 (Browning et al. 2007). Remarkably, the binding of two structurally and chemically distinct ligands, ponA and DBH, resulted in *Heliothis* EcR adopting a different conformation. In particular, the corresponding ligand-binding pockets were found to only partially overlapped and displayed different sizes and shapes (Billas et al. 2003). For EcRLBD structures, the β-sheet forming ligand-binding pocket is composed of three strands. This is a key region of the receptor that displays unusual flexibility in response to different ligand types. For synthetic DBH agonists, a complete rearrangement of the region of the β-sheet was observed, involving two aromatic residues, Phe397 and Tyr403, that changed con-

formation to fill the space left unoccupied by the small DBH molecule (Billas et al. 2003). Another critical region is the connection between helices H1–H3, which in 20E- and ponA-bound EcR structures features a small helix H2 that is absent in the complex with the DBH compound (Billas et al. 2003). The existence of this small helix seems to be closely linked to the existence of direct and indirect stabilizing interactions with the ligand. Because the small DBH molecule is not located in close vicinity to the region comprising helix H2 and the β-sheet, H2 is not ligand-mediated and unfolds into a loop (Billas et al. 2003; Billas and Moras 2005). All this crystallographic data indicate that the ligand binding domain of EcR is characterized by unusual high degree of flexibility. As mentioned above, EcRDBD also displays flexibility and adaptability. The EcRLBD/UspLBD heterodimer interface is comprised of an intricate network of hydrophobic and polar interactions mediated in both partners by the helices H7, H9, H10 and the loop connecting helices H8 and H9 (L8-9). The interface of the *Heliothis* EcRLBD/UspLBD differs from that of the *Bemisia* EcRLBD/UspLBD or the *Tribolium* EcRLBD/UspLBD. In the *Heliothis* EcRLBD/UspLBD, the heterodimeric arrangement is more compact than in the *Bemisia* and *Tribolium* EcRLBD/UspLBD, due to the concomitant movement of structural elements from both partners and an additional zone that contributes to the heterodimerization interface involving of EcR H7 and Usp L8-9 (Iwema et al. 2007). The dimerization interface observed for *Bemisia* and *Tribolium* EcRLBD/UspLBD is similar to interface observed for RXR in vertebrate NR heterodimers, where RXR L8-9 does not contribute to heterodimer contacts (Iwema et al. 2009),

The structure of *Heliothis* Usp in the complex (Browning et al. 2007) is almost identical to the structure of the monomer (Billas et al. 2001), with a large hydrophobic ligand-binding pocket that is filled with a phospholipid molecule. A similar X-ray structure was also observed for the *Drosophila* UspLBD (Clayton et al. 2001). These structures are significantly different in comparison to human RXRLBD structures (Egea et al. 2000), the activation helix H12 is locked in a so-called antagonist conformation by intra-protein interactions with a structural motif conserved in Mecopterida Usps (Clayton et al. 2001; Browning et al. 2007). A structure without a ligand-binding pocket was reported for the *Bemisia* UspLBD (Carmichael et al. 2005) and the *Tribolium* UspLBD (Iwema et al. 2007). The LBPs are filled by residues that stabilize an apo conformation and H12 is positioned in a so-called antagonist conformation, (Carmichael et al. 2005; Iwema et al. 2007). The structure of the ligand-binding pocket of Usp was highly plastic during insect evolution, adopting three of the four states known to NRs: a nutritional sensor (basal insects), a real orphan (*Hemiptera, Coleoptera*) and a receptor with constitutive activity (*Mecopterida*) (Chaumot et al. 2012). Maintenance of the ecdysone pathway was achieved, at least in part, through the molecular adaptation of the UspLBD (Iwema et al. 2007). Some experimental data suggest that JH is the Usp ligand (Jones and Sharp 1997; Xu et al. 2002).

3 Structural Analysis of Disordered Regions

In addition to the highly evolutionarily conserved, globular DBD and LBD, which have a stable fold, there are also IDRs, i.e. the A/B region called the NTD which is hypervarible in sequence and length, the D hinge region linked to the DBD and F region which only a few NRs possess (McEwan 2009). IDRs and intrinsically disordered proteins (IDPs) are characterized by the lack of a stable and unique three-dimensional structure under physiological conditions. They are able to adopt distinct structural features upon interaction with particular protein partners or small molecular ligands (Uversky 2002; Wright and Dyson 1999). Additionally, their binding functions can be altered by posttranslational modifications (PTMs) (Uversky et al. 2008). Structural intrinsic disorder (ID) is thought to be essential to these proteins, as their various biological functions stem either directly from this state or from some local folding or ordering during molecular recognition (Tompa 2002). Comprehensive bioinformatic analysis provided evidences of ID in NR families (Krasowski et al. 2008). The ID content of nearly 400 NRs in particular regions or domains was calculated across the full sequence. The NTD and hinge region was found to have a nearly 2.7 times higher probability of ID than the LBD across all species.

Despite the fact that there have been extensive studies on the structure and function of the arthropod functional ecdysteroid receptor and its partners in 20E signalling, there have been only three reports detailing the molecular characteristics of the NTDs isolated from insects: the EcRNTD (Nocula-Ługowska et al. 2009) and the DHR38NTD from *Drosophila* (Dziedzic-Letka et al. 2011) and the UspNTD from *Aedes aegypti* (Pieprzyk et al. 2014). There is only one report on the *Drosophila* EcR hinge region (Zoglowek et al. 2012) and nothing is known about the F region. One of the three reports thoroughly describes the structural properties of the *Drosophila* EcRNTDs (Nocula-Ługowska et al. 2009). In *Drosophila* there are three EcR isoforms, EcRA, EcRB1, and EcRB2 that exhibit diverse spatial and temporal distributions within various tissues and reveal important functional differences (Talbot et al. 1993). Recently the structural properties of the NTDs of EcRA and EcRB1 isoforms have been reported. *In silico* analysis performed using different bioinformatic tools showed the existence of large IDRs in both NTDs. Moreover, analysis done with various techniques, e.g. CD spectroscopy or size-exclusion chromatography showed that both NTDs had features of the collapsed, disordered conformation that resembles that of pre-molten (PMG)-like IDPs (Nocula-Ługowska et al. 2009). However, the analysed NTDs have distinct structural properties. In particular, the *Drosophila* EcRANTD has a lower content of a regular secondary structure than the EcRB1NTD and the EcRANTD is less compact than the EcRB1NTD. It has been previously reported that these isoforms reveal different transcription activities depending on the promoter used and the type of host cells (Dela Cruz et al. 2000; Hu et al. 2003; Mouillet et al. 2001). It has been postulated that the functional differences reported for the EcRA and EcRB1 isoforms might be the result of subtle structural differences that were observed in their NTDs (Nocula-Ługowska et al.

2009). Dziedzic-Letka *et al.* (Dziedzic-Letka et al. 2011) also studied the structural characteristics of the NTD, but from another member of the NR family from *Drosophila,* DHR38 (Dziedzic-Letka et al. 2011). Studies on the function of DHR38 indicated that this NR is involved in 20E (Baker et al. 2003; Sutherland et al. 1995). It was suggested that the DHR38NTD may be involved in some protein-protein interactions that are critical for the transmission of an ecdysteroid-dependent signalling pathway (Dziedzic-Letka et al. 2011). Despite the fact that the DHR38NTD has an amino acid composition that is different from that of the EcRNTDs, both, bioinformatics analysis and comprehensive biophysical and biochemical analyses showed that the DHR38NTD exhibited characteristics reminiscent of a PMG-like IDP with a partially unfolded conformation and regions of secondary structures, similarly as was observed for the EcRNTD. Moreover, size-exclusion chromatography in denaturing conditions and CD spectroscopy in the presence of osmolytes, which are known to promote a function-related local structure, showed that the DHR38NTD could adopt a disordered state or more ordered conformations in response to changes in environmental conditions. It was suggested that the structure of the DHR38NTD could have been influenced by multiple phosphorylation that promoted and mediated distinct signalling effects. Because of the formation of different conformational states, many distinct interaction surfaces might be formed and recognized by specific protein partners (Dziedzic-Letka et al. 2011). Further detailed studies are necessary to see if phosphorylation can indeed induce conformational changes in the DHR38NTD. Recently, increasingly more attention has been paid to UspNTD. In the mosquito *Aedes aegypti* two Usp isoforms, UspA and UspB, have been cloned and identified (Kapitskaya et al. 1996; Wang et al. 2000). Recently biochemical and biophysical properties of the UspBNTD have been described (Pieprzyk et al. 2014). The results showed that the UspBNTD is an IDP which has residual secondary structures. The anomalous behaviour of the UspBNTD in SDS-PAGE electrophoresis and in secondary structure analyses by far-UV CD spectroscopy and size exclusion chromatography indicated features of an IDP. However, in contrast to the *Drosophila* EcRANTD, EcRB1NTD and DHR38NTD which were reported to have been PMG-like IDPs, the *Aedes* UspBNTD cannot be unequivocally classified into one of the recognized classes of IDPs, based on its amino acid composition, molecular mass, Stokes radius and secondary structure content. Nevertheless, the sedimentation velocity analytical ultracentrifugation experiment determined that *Aedes* UspBNTD had the shape of an asymmetric, elongated and elliptically-shaped protein. Similarly to the NTDs described above, the UspBNTD has the potential to form structures in the presence of a folding/refolding agent. This again proves the assumption that these NTDs have the ability to exist in a wide range of conformational states and may have the ability to bind to several different partner proteins, thus exhibiting multiple functions as with other IDPs (Wright and Dyson 1999). What is particularly noteworthy and what makes the *Aedes* UspBNTD radically different from other insect NTDs that have been described is the fact that the UspBNTD exhibited a tendency for homooligomerization and probably coexists in solution as a monomeric and dimeric species (Pieprzyk et al. 2014). A comparison was made of the propensity for oligomeric forms in

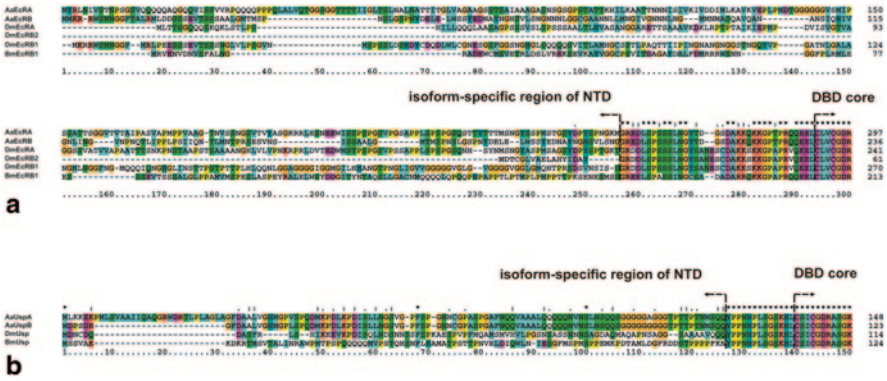


Fig. 4 Alignment of the NTD sequences of selected insect species. **a** Amino acid alignment of the N-terminal domain of EcR from *Aedes aegypti* (UniProtKB: P49880), *Drosophila melanogaster* (P34021), *Bombyx mori* (P49881); **b** Amino acid alignment of the N-terminal domain of Usp from *A. aegypti* (Q9GSG8; UspBNTD: Q9GSG7), *D. melanogaster* (P20153), *B. mori* (H9J9J0). The dots indicate conservation between groups of weakly similar properties; colons indicate conservation between groups of strongly similar properties and asterisks indicate positions which have a single, fully conserved residue. All alignments were done using ClustalX (Thompson et al. 1997)

other insect NTDs, namely the NTD of Usp from *Drosophila* and the NTD of Usp from *Bombyx mori*. The dimerization potential is conserved among these receptors, but to a different extent within each species. The *Aedes* UspBNTD exhibits the highest tendency to form a dimer, then the homologs from *Drosophila* and *Bombyx*. Rymarczyk *et al.* (Rymarczyk et al. 2003) suggested that the *Drosophila* UspNTD probably plays an important role in stabilizing the receptor oligomers in solution and may possess an additional interface for dimer interaction or may be a part of existing interfaces within the full-length protein (Rymarczyk et al. 2003). Pieprzyk *et al.* (Pieprzyk et al. 2014) suggested that the NTDs from *Aedes, Drosophila* and *Bombyx* may have different structural architecture and that the specifics that determine dimerization might be distributed differently within each domain (Pieprzyk et al. 2014). Despite differences in the amino acid sequences (Fig. 4), all the insect NTDs described above exhibit properties of IDP molecules and have a pliable structure. They were not completely random coil-like, since there was content of a residual secondary structure. It is worth emphasizing that although they were all classified as IDRs, there are subtle differences in their structure, like a different degree of secondary structure or compactness or differences in hydrodynamic properties any of which may result in different activity (Pieprzyk et al. 2014). Moreover, the NTDs may be involved in dynamic protein-protein interactions through their ability to adopt distinct conformations. They may also regulate mechanisms in signal transduction, e.g. by having different tendencies for dimerization. IDPs and IDRs, while structurally poor, are functionally rich by virtue of their flexibility and modularity (Malaney et al. 2013). There is evidence indicating an intimate relationship between PTMs and structural disorder (Gao and Xu 2012). These modifications involve low affinity, high specificity binding interactions between a specific enzyme and the protein that is modified (Xie et al. 2007). Based on observed correlations between

PTMs and predicted disorder, it was shown e.g. that IDPs/IDRs are substrates of twice as many kinases as ordered proteins are (Uversky et al. 2008). By comparing a collection of more than 1500 experimentally determined S, T, and Y phosphorylation sites to potential sites that were nonphosphorylated, the segments surrounding phosphorylation sites were found to be significantly enriched in amino acids that are usually in IDRs (Iakoucheva et al. 2004). The majority of kinases, whose substrates were mostly IDPs/IDRs, were either regulated in a manner that is dependent on the stage of the cell division cycle or were activated upon exposure to particular stimuli or stress. Therefore, protein modifications may not only serve as important regulatory mechanisms that fine-tune the functions of IDPs or IDRs, but they may also be necessary to tightly control the availability of these proteins under different conditions (Uversky et al. 2008).

Relatively little work has focused on the PTMs of insect NRs. Phosphorylation was shown to play an important role in regulating the function of the EcR/USP complex (Sun and Song 2006). Both EcR and Usp are phosphoproteins and their phosphorylation is regulated by 20E (Song and Gilbert 1998; Rauch et al. 1998; Nicolai et al. 2000). However, only the site of modification in *Drosophila* Usp was identified. Using liquid chromatography-tandem mass spectrometry, the site of protein kinase C (PKC) phosphorylation was found to be located in the NTD (S35) (Wang et al. 2012) . BLAST search against the NCBI protein database revealed that S35 of Usp is well conserved in other *Diptera.* However, there is no information about how this modification impacts the structure of the NTDs. Additionally, nothing is known about the sites of EcR phosphorylation. Bioinformatics tools indicated that putative phosphorylation sites are present mainly in the NTD (Rauch et al. 1998), but this requires experimental verification.

Sumoylation is deeply involved in PTMs and has a significant influence on the structure of NR NTDs. This is a protein conjugation resembling ubiquitination, based on the reversible attachment of the small ubiquitin-related modifier (SUMO) protein (Geiss-Friedlander and Melchior 2007). At least some insect NRs have the SUMO peptide added to a K residue in their NTDs. *Drosophila* EcR undergoes isoform-specific multi-sumoylation. The pattern of modification remains unchanged in the presence of the ligand and the dimerization partner. The SUMO acceptor sites are located in the NTD, DBD, LBD and region F. However, the most interesting modification site is in the NTD as it encompasses only isoform A (Seliga et al. 2013). This was also in agreement with what had been predicted with bioinformatics tools (Watanabe et al. 2010). A comprehensive structural comparison revealed that the A isoform-specific region of EcR from different species of arthropods contained evolutionally conserved microdomain structures including the sumoylation motif. Sumoylation of the A isoform-specific region might contribute to transcriptional regulation that is either cell-type or organism dependent and mediated by the NTD of the EcR A isoform (Watanabe et al. 2010). The consequences of modification and the resulting impact on conformation and function may be especially crucial for the disordered sequences in these areas. Sumoylation may influence the structure and mode of action both EcR and its partner, Usp. The main sites of sumoylation of *Drosophila* Usp are located in the NTD (Bielska et al. 2012), where three K residues can

be sumoylated, but the attachment of one SUMO molecule prevents modification of the other two remaining sites. One possible explanation for this phenomenon could be steric hindrance, as all K residues in the NTD lie close to each other and the attachment of one SUMO molecule can apparently block the modification of others. However, monosumoylation of the NTD is most likely caused by interaction with proteins which can lock the NTD region in a specific conformation, exposing one of the three K residues to SUMO modification. Alternatively, monosumoylation might be a consequence of conformational changes after SUMO modification. It has been suggested that the sumoylation of Usp could be an important factor that modulates its activity by changing inter- and intra-molecular interactions (Bielska et al. 2012). Interestingly, the amino acid sequence alignment of the NTDs of Usp from *Diptera*, *Hymenoptera*, *Coleoptera* and *Hemiptera* showed that the K20 residue identified as a SUMO acceptor site and the embedded sequence in *Drosophila* Usp in the NTD is highly conserved in other classes of insects. This suggests that the K20 residue constitutes an important regulatory element of the transcriptional activity of Usp receptors (Bielska et al. 2012).

Another IDR identified in NRs is the C-terminus of the LBD called the F region, which was mentioned above. This region has few structural features, displays little evolutionary conservation and reveals no clues as to the function of the sequence (Hu et al. 2003). It had been suggested that the F region might play a role in recruiting a co-activator in the E domain and in determining the specifics of the LBD co-activator interference (Laudet 2002). The F region of EcR from different species consists largely of repeated residues, and there is no apparent conservation of its sequence. *Drosophila* EcR contains an unusually long F region (223 residues), for which there is no known function. By contrast the C-terminal region in EcRs from other flies are significantly shorter—most NRs contain an F region of 10–50 residues (Hu et al. 2003). The F region of insect NRs, similarly to the NTD, is also a target of PTMs. The K residue in the F region of each *Drosophila* EcR isoform (K871, K842 and K662, respectively for EcRB1, EcRA and EcRB2) was shown to be subjected to sumoylation (Seliga et al. 2013). Due to the variability in length and sequence among EcRs, it is difficult to assume that EcRs from species other than *Drosophila* might be sumoylated in F region.

Drosophila Usp was also shown to be modified by SUMO in the F region (K506) (Bielska et al. 2012). The F region was analyzed together with the E region, because the F region has only 12 aa and is too short to be independently investigated. In the isolated E/F region of Usp there are two alternatively sumoylated residues: K424 (E region) and K506 (F region). However, these residues are not available for sumoylation in the full-length protein. The main sites of sumoylation of full-length Usp are located in the NTD, although sumoylation of some residues in E or F regions is also possible (Bielska et al. 2012). This adds another level of complexity to NR signal integration, since PTMs might influence the properties of the encoded functions.

The roles of phosphorylation, sumoylation and others modifications of EcR and Usp are beginning to be elucidated, but the possible impact on structure is less clear. The continuing challenge is to determine the functional and/or structural

significance of individual modifications, and to determine whether such modifications may actually act co-operatively to regulate receptor function.

4 New Targets for Structural Analysis

NRs can quickly elicit a response for a wide range of molecular compounds starting from steroid hormones, retinoids, through dietary lipids and many other ligands. This action is subjected to regulation by a cohort of co-regulators, mainly co-repressors and co-activators as well as chaperons, which often form heterocomplexes. Co-regulators can modulate chromatin or can directly bind to NRs, changing their conformation and resulting in repression or promotion of transcription. Exactly how co-regulators are involved in transcription processes is an interesting and delicate issue. There are already a number of recognized co-regulators of NRs and the number is still growing (McEwan 2009). A good example which illustrates how these factors can alter or influence the function of NRs are the regulatory proteins which are part of the transcriptional machinery for genes under the control of 20E. In *Drosophila,* the functional ecdysone receptor formed by EcRB1 and Usp undergoes hormone binding activity but is unable to bind EcRE. To bind the DNA, the heterodimer requires activation by a chaperone (Arbeitman and Hogness 2000). What is more, the function of the active ecdysone receptor is influenced by the action of JH. During the larval development of insects, JH modulates 20E action which prevents metamorphosis. For this reason, JH is referred to as the "status quo" hormone (Riddiford 1996). 20E and JH signaling pathways interact to mediate insect development, but the mechanism of this cross-talk is poorly understood (Jindra et al. 2013). Recent findings suggest that the signaling by 20E and JH involves the same co-regulators to mediate the cross-talk between the two hormonal signaling pathways (Li et al. 2007; Liu et al. 2011).

Although a great deal is known about the biological and molecular action of 20E, the mechanism of the function of JH remains a puzzle. Perhaps because of its unique, sesquiterpenoid structure, the search for the JH receptor has been long and difficult (Riddiford 2008). Currently, there is inconclusive experimental evidence to determine whether the JH-dependent induction of gene expression requires only hormone binding to the cell surface or whether induction is mediated through a receptor-dependent mechanism which requires hormone entry into the cell (Li et al. 2007). Many attempts have been made to identify the JH receptor. The first candidate for the JH receptor was Usp (Jones and Sharp 1997). However, more detailed studies showed that JH III binding for the Usp receptor is more than 100 times weaker than expected for a NR (Jones et al. 2001). Jones and Jones (Jones et al. 2006) suggested a number of experimental approaches to learn about possible JH action by Usp *in vivo* (Riddiford 2008) Another candidate for the JH receptor is methoprene-tolerant protein (Met) found in *Drosophila* (Wilson and Fabian 1986; Shemshedini and Wilson 1990). The *met* gene product presents high-affinity JH binding (Miura et al. 2005; Shemshedini and Wilson 1990), expression in JH

target tissues (Pursley et al. 2000; Liu et al. 2009) and JH-dependent transcriptional activity (Miura et al. 2005). The Met mutants are resistant to the toxic and morphogenetic effects of JH and JH agonist insecticides, such as methoprene (Wilson and Fabian 1986; Shemshedini and Wilson 1990). However, the hypothesis that Met is the JH receptor did not meet expectations when Met null mutants were viable (Wilson and Ashok 1998) rather than being a lethal phenotype (Riddiford 2008). The explanation came from the analysis of the sequenced *Drosophila* genome when the second gene of the basic helix-loop-helix (bHLH-PAS) protein, which is closely related to Met with 68–86% identity in conserved regions, was found—the germ cell-expressed (Gce) (Moore et al. 2000). The presence of overexpressed Gce can partially substitute for Met in flies (Baumann et al. 2010) and Gce binds JH with higher affinity than Met (Charles et al. 2011). Met was shown to form homodimers and heterodimers with Gce in the absence of JH, while the presence of JH blocked dimerization (Godlewski et al. 2006). The search for Met homologs in other insects identified the single ortholog of *Drosophila* Met and Gce in three mosquito species, *Culex pipiens, A. aegypti, Anopheles gambiae* (Wang et al. 2007) and in coleopteran, *T. castaneum (Konopova and Jindra* 2007*)*. Li et al. (Li et al. 2010) identified both *met* and *gce* genes in *Bombyx* and suggested using the Gce name instead of Met in insects with a single orthodolog (Konopova and Jindra 2007). Afterwards, however, Guo *et al*. (Guo et al. 2012) referred to both genes in *Bombyx* as *met1* and *met2* (Guo et al. 2012). Met in *Bombyx* and *Drosophila* species might have originated from an ancestor gene with *gce* by gene duplication (Li et al. 2010). Met and Gce are members of the bHLH-PAS family of transcription factors (Ashok et al. 1998). The bHLH domain consists of the basic domain, encompassing approximately 15 aa with a high number of basic residues, followed by two amphipathic α-helices and a variable length loop region between them. Interaction between the helix regions of two different proteins leads to their dimerization, and the basic region of each partner binds to the half-site of the E-box CANNTG to regulate transcription (Moore et al. 2000). Despite the enormous regulatory diversity provided by the heterodimeric structure of bHLH complexes, variations in the sequence of the DNA-binding site are not sufficient to account for the capacity to induce specific gene expression. PAS domains exist in many signalling proteins and are generally used as a signal sensor domain responsible for specific protein-protein interaction. The PAS region in bHLH-PAS proteins consists of two adjacent PAS domains, degenerate repeats of proximity 130 amino acids, termed PAS A and PAS B, forming a highly conserved structure, despite having low primary sequence homology. PAC motifs occur in the C-terminal in a subset of all known PAS motifs and are thought to contribute to the PAS domain fold. The contribution of the PAS domain to the selection of target genes was examined by constructing the Trh–Sim PAS chimera, in which the PAS domain of Sim was used to replace that of Trh. The results showed that specificity is indeed determined by the PAS domain. Met and Gce possess two conserved PAS motifs, PAS A and PAS B, that are a hallmark feature of the bHLH-PAS proteins (Bernardo and Dubrovsky 2012a). To understand how the structure of Met might accommodate the hormonal ligand, Charles *et al.* (Charles et al. 2011) modelled the *Tribolium* Met PAS-B domain and documented findings that the PAS-B domain was

sufficient and necessary for JH binding by Met (Charles et al. 2011). Interestingly, the PAS-B of Met contained one of the Met subcellular localization signals—the JH dependent nuclear import signal (NLS) (Greb-Markiewicz et al. 2011). It seems that binding of JH elicits a conformational change that overrides the homophilic bonds between the bHLH, PAS-A, and PAS-B domains of unliganded Met molecules and makes Met accessible to other proteins (Jindra et al. 2013). *Drosophila* Taiman, *Aedes* FISC, and *Tribolium* SRC interact with Met in a JH dependent manner through their PAS domains (Li et al. 2010, Zhang et al. 2011). NR FTZ-F1 interacts with the non-canonical LIXXLL motif in the C-terminal part of Gce and Met in the presence of JH. Deletion of the PAS-B domain disrupted the interaction even when the NR box was intact, indicating that the NR box may be functional only when Met is in an active, JH-bound conformation (Bernardo and Dubrovsky 2012b). A common feature of bHLH-PAS proteins is the presence of acidic, proline/serine (P/S), and glutamine-rich (Q_R) sequences in the C terminus, which could serve as transactivation domains. Functional divergence is evident in the C-terminus of Met and Gce. In addition to the non-canonical NR box, the Q_R region of Met is used as a secondary NR interaction site by SRC family members (Bernardo and Dubrovsky 2012b). JH activation of the NR E75A gene requires Gce but appears not to require Met; this represents the first example of an endogenous JH-dependent transcription mediated by Gce receptors that has been found, showing that the JH activation of E75A also requires an orphan receptor, FTZ-F1 (Dubrovsky et al. 2011). While it appears that both Met and Gce function as JH receptors, more detailed analysis is needed along with empirical research on the structure of both proteins (Bernardo and Dubrovsky 2012b).

A different approach was used to identify the JH receptor by attempting to identify the potential JH response element. In 2007, a common 29-nucleotide JH-response element (JHRE) was identified in the promoter regions of 13 out of 16 genes regulated by JH in *D. melanogaster* L57 and *Apis mellifera* brain cells (Li et al. 2007). In addition, two nuclear proteins, 21 kDa calponin-like protein (Chd64) and 39 kDa FK506-binding protein (FKBP39) were found to bind to JHRE as well as some other nuclear proteins including: EcR, Usp and Met. Suppression of the induction of Chd64 and FKBP39 by specific dsRNA prevented JH induction of the JHRE-dependent reporter, suggesting that these proteins are necessary for JH action. Based on their findings, the researchers proposed a model in which Chd64 and FKBP39 are part of a multi-protein complex that mediates the cross-talk between JH and 20E. Following this model, in the presence of a high level of JH and 20E (larval molting stage), Chd64 and FKBP39 interact with EcR, Usp, and other cofactors. This complex can bind to the JHRE, causing an increase in the expression of genes containing JHRE and a decrease in the expression of ecdysone - response genes. During metamorphosis, in the absence of JH, levels of Chd64 and FKBP39 are low. Then, EcR heterodimerizes with other members of the NR superfamily, leading to expression of ecdysone-response genes (Li et al. 2007). However, this model is not perfect and is oversimplified in some aspects (Riddiford 2008). Although the model representing the mode of action of Chd64 and FKBP39 is only hypothetical and requires further research, it is an interesting approach. Until now

there has been a lack of information on the exact mode of action of Chd64 and FKBP39, and the mechanism of interactions with JHRE and other proteins remain unknown. Neither Chd64 nor FKBP39 are typical DNA-binding proteins or typical signal transducers. A great deal of work needs to be done in order to understand the role of these two proteins in multiple and dynamic protein complexes.

The currently available structural information is presented below on Chd64 and FKBP39, which may provide insight into their ability to bind JHRE and at the same time interact with many proteinous partners. This is what could possibly make them key players in the cross-talk between 20E and JH. Chd64 belongs to a family of proteins that contain a calponin homology (CH) domain. The CH domain is a highly conserved animal module built by approximately 100 invariant core residues (Gimona and Mital 1998; Banuelos et al. 1998). The family members differ in the number of CH repeats and the presence of additional modules, e.g. the EF hand or calponin-family repeat (CFR) (McGough 1998; Hartwig 1995). In terms of topology, Chd64 is related to cytoskeletal transgelin, a protein that contains one CH domain and a single CFR. In addition to actin binding activity, transgelin has been shown to be involved in other cellular processes (Wilson et al. 2006), e.g. to prevent binding of an androgen receptor with its co-activator (Yang et al. 2007) or to suppress expression of the metallo-matrix proteinase by prohibiting the trans-activation of the promoter (Nair et al. 2006). Interestingly, insect transgelin-like HaCal from *Helicoverpa armigera* has been shown to maintain a cross-talk between 20E and JH (Liu et al. 2011).

Until now very little has been known about the molecular bases of the interactions which maintain the regulatory function of the CH domain containing proteins (Banuelos et al. 1998; Gimona and Mital 1998; Gimona et al. 2002). Interestingly, in a recent paper the disorderness in Chd64 from *Drosophila* and *Tribolium* has been identified (Kozłowska et al. 2014). *In silico* analyses by a range of disorder predictors revealed that the core of the protein which corresponds to the probable localization of the putative CH domain is globular, whereas the remaining fragments appear to be disordered. Terminal IDRs are perfect candidates for being a platform for multiple interactions (Kozłowska et al. 2014). Such regions are often involved in regulatory processes, since they provide larger interaction surfaces when compared to globular proteins of similar length (Dunker et al. 2001). Their flexibility and the exposure of short linear peptides enable disordered proteins to interact with numerous and various partners, including other proteins, nucleic acids, membranes or small molecules (Dunker et al. 2005). In addition, disorder is more often observed on protein termini than in the center of a polypeptide chain and the functional importance of multi-tasking disordered protein termini has recently been emphasized (Uversky 2013).

The other important ecdysone receptor that modulates protein and is probably a key player in the cross-talk between 20E and JH in *Drosophila* is immunophilin FKBP39 (Li et al. 2007). Immunophilins are a family of proteins that can bind the immunosuppressive drug FK-506 (Galat 2003). The main domain, the FKBD (FK506 binding domain), consists mostly of β-sheets which form an extremely hydrophobic pocket which is responsible for maintaining many regulatory processes

(Van Duyne et al. 1993). This is mostly due to its peptidyl prolyl *cis-trans* isomerase (PPIase) activity. PPIases induce conformational changes in proteins through the conversion of proline bonds from *cis* to *trans,* which is a rate limiting step in protein folding (Schmid 1993). *Drosophila* FKBP39 has a C-terminal FKBD domain and its central and N-terminal is composed of acidic and basic regions. This kind of structural organization in nuclear FKBPs is repeatable in many insect species, suggesting that there are functional similarities. The possibly negatively and positively charged regions may allow interaction with positively charged nuclear proteins, such as histones and acidic molecules i.e. DNA (Himukai et al. 1999). The FKBD domain has been shown to carry a nuclear localization signal in some FKBP39 homologues and was postulated to be necessary for correct folding of the protein to translocate to the nucleus (Alnemri et al. 1994).

New targets have emerged in the latest research on NRs. They include a wide range of ligands, proteins, or their complexes or even the whole signaling pathways of non-related hormones. Often only a complex approach can make it possible to conduct in-depth and detailed analysis of subtly precise physiological gene regulation by means of NRs. There is a plenty of scientific work to be done in order to identify and describe the modes of action. Chd64, FKBP39 and Met are good examples. They require intensive experimental characterization on a molecular level in order to elucidate all the aspects of their role in the cross-talk between 20E and JH, including binding DNA, interaction with each other and other components of multi-protein complexes. Understanding their exact physiological role and mode of action brings new research challenges on the function of the functional ecdysteroid receptor.

Acknowledgement This work was supported by National Science Centre grant (2012/05/B/NZ1/00659).

References

Aagaard MM, Siersbaek R, Mandrup S (2011) Molecular basis for gene-specific transactivation by nuclear receptors. Biochim Biophys Acta 1812:824–835

Alnemri ES, Fernandes-Alnemri T, Pomerenke K, Robertson NM, Dudley K, Dubois GC, Litwack G (1994) FKBP46, a novel Sf9 insect cell nuclear immunophilin that forms a protein-kinase complex. J Biol Chem 269:30828–30834

Antoniewski C, Laval M, Dahan A, Lepesant JA (1994) The ecdysone response enhancer of the Fbp1 gene of Drosophila melanogaster is a direct target for the EcR/USP nuclear receptor. Mol Cell Biol 14:4465–4474

Antoniewski C, Mugat B, Delbac F, Lepesant JA (1996) Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in Drosophila melanogaster. Mol Cell Biol 16:2977–2986

Arbeitman MN, Hogness DS (2000) Molecular chaperones activate the Drosophila ecdysone receptor, an RXR heterodimer. Cell 101:67–77

Ashok M, Turner C, Wilson TG (1998) Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc Natl Acad Sci USA 95:2761–2766

Baker KD, Shewchuk LM, Kozlova T, Makishima M, Hassell A, Wisely B, Caravella JA, Lambert MH, Reinking JL, Krause H (2003) The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. Cell 113:731–742

Banuelos S, Saraste M, Djinovic-Carugo K (1998) Structural comparisons of calponin homology domains: implications for actin binding. Structure 6:1419–1431

Baumann A, Barry J, Wang S, Fujiwara Y, Wilson TG (2010) Paralogous genes involved in juvenile hormone action in Drosophila melanogaster. Genetics 185:1327–1336

Bernardo TJ, Dubrovsky EB (2012a) Molecular mechanisms of transcription activation by juvenile hormone: A critical role for bHLH-PAS and nuclear receptor proteins. Insects 3:324–338

Bernardo TJ, Dubrovsky EB (2012b) The Drosophila juvenile hormone receptor candidates methoprene-tolerant (MET) and germ cell-expressed (GCE) utilize a conserved LIXXL motif to bind the FTZ-F1 nuclear receptor. J Biol Chem 287:7821–7833

Bielska K, Seliga J, Wieczorek E, Kedracka-Krok S, Niedenthal R, Ożyhar A (2012) Alternative sumoylation sites in the Drosophila nuclear receptor Usp. J Steroid Biochem Mol Bio 132:227–238

Billas IM, Moras D (2005) Ligand-binding pocket of the ecdysone receptor. Vitam Horm 73:101–129

Billas IM, Moulinier L, Rochel N, Moras D (2001) Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. J Biol Chem 276:7465–7474

Billas IM, Iwema T, Garnier JM, Mitschler A, Rochel N, Moras D (2003) Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. Nature 426:91–96

Browning C, Martin E, Loch C, Wurtz JM, Moras D, Stote RH, Dejaegere AP, Billas IM (2007) Critical role of desolvation in the binding of 20-hydroxyecdysone to the ecdysone receptor. J Biol Chem 282:32924–32934

Carmichael JA, Lawrence MC, Graham LD, Pilling PA, Epa VC, Noyce L, Lovrecz G, Winkler DA, Pawlak-Skrzecz A, Eaton RE, Hannan GN, Hill RJ (2005) The X-ray structure of a hemipteran ecdysone receptor ligand-binding domain: comparison with a lepidopteran ecdysone receptor ligand-binding domain and implications for insecticide design. J Biol Chem 280:22258–22269

Charles JP, Iwema T, Epa VC, Takaki K, Rynes J, Jindra M (2011) Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proc Natl Acad Sci USA 108:21128–21133

Chaumot A, Da Lage JL, Maestro O, Martin D, Iwema T, Brunet F, Belles X, Laudet V, Bonneton F (2012) Molecular adaptation and resilience of the insect's nuclear receptor USP. BMC Evolut Bio 12:199-2148-12-199

Cherbas L, Lee K, Cherbas P (1991) Identification of ecdysone response elements by analysis of the Drosophila Eip28/29 gene. Genes Dev 5:120–131

Clayton GM, Peak-Chew SY, Evans RM, Schwabe JW (2001) The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. Proc Natl Acad Sci USA 98:1549–1554

D'Avino PP, Crispi S, Cherbas L, Cherbas P, Furia M (1995) The moulting hormone ecdysone is able to recognize target elements composed of direct repeats. Mol Cell Endocrinol 113:1–9

Dela Cruz FE, Kirsch DR, Heinrich JN (2000) Transcriptional activity of Drosophila melanogaster ecdysone receptor isoforms and ultraspiracle in Saccharomyces cerevisiae. J Mol Endocrinol 24:183–191

Delano WL 2002. The PyMOL user's manual. DeLano Scientific, San Carlos

Devarakonda S, Harp JM, Kim Y, Ozyhar A, Rastinejad F (2003) Structure of the heterodimeric ecdysone receptor DNA-binding complex. EMBO J 22:5827–5840

Dobryszycki P, Grad I, Krusiński T, Michaluk P, Sawicka D, Kowalska A, Orłowski M, Jakób M, Rymarczyk G, Kochman M, Ożyhar A (2006) The DNA-binding domain of the ultraspiracle drives deformation of the response element whereas the DNA-binding domain of the ecdysone receptor is responsible for a slight additional change of the preformed structure. Biochemistry 45:668–675

Dubrovsky EB, Dubrovskaya VA, Bernardo T, Otte V, Difilippo R, Bryan H (2011) The Drosophila FTZ-F1 nuclear receptor mediates juvenile hormone activation of E75A gene expression through an intracellular pathway. J Biol Chem 286:33689–33700

Dummitt B, Chang YH (2006) Molecular beacons for DNA binding proteins: an emerging technology for detection of DNA binding proteins and their ligands. Assay Drug Dev Technol 4:343–349

Dunker AK, Lawson JD, Brown CJ, Williams RM, Romero P, Oh JS, Oldfield CJ, Campen AM, Ratliff CM, Hipps KW, Ausio J, Nissen MS, Reeves R, Kang C, Kissinger CR, Bailey RW, Griswold MD, Chiu W, Garner EC, Obradovic Z (2001) Intrinsically disordered protein. J Mol Gr Model 19:26–59

Dunker AK, Cortese MS, Romero P, Iakoucheva LM, Uversky VN (2005) Flexible nets. The roles of intrinsic disorder in protein interaction networks. FEBS J 272:5129–5148

Dutko-Gwóźdź J, Gwóźdź T, Orłowski M, Greb-Markiewicz B, Dus D, Dobrucki J, Ożyhar A (2008) The variety of complexes formed by EcR and Usp nuclear receptors in the nuclei of living cells. Mol Cell Endocrinol 294:45–51

Dziedzic-Letka A, Rymarczyk G, Kapłon TM, Górecki A, Szamborska-Gbur A, Wojtas M, Dobryszycki P, Ożyhar A (2011) Intrinsic disorder of Drosophila melanogaster hormone receptor 38 N-terminal domain. Proteins 79:376–392

Egea PF, Mitschler A, Rochel N, Ruff M, Chambon P, Moras D (2000) Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. EMBO J 19(11):2592–601

Fang X, Li JJ, Tan W (2000) Using molecular beacons to probe molecular interactions between lactate dehydrogenase and single-stranded DNA. Anal Chem 72:3280–3285

Fisk GJ, Thummel CS (1995) Isolation, regulation, and DNA-binding properties of three Drosophila nuclear hormone receptor superfamily members. Proc Natl Acad Sci USA 92:10604–10608

Galat A (2003) Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity–targets–functions. Curr Top Med Chem 3:1315–1347

Gao J, Xu D (2012) Correlation between posttranslational modification and intrinsic disorder in protein. Pac Symp Biocomput 94–103

Geiss-Friedlander R, Melchior F (2007) Concepts in sumoylation: a decade on. Nat Rev Mol Cell Bio 8:947–956

Gimona M, Mital R (1998) The single CH domain of calponin is neither sufficient nor necessary for F-actin binding. J Cell Sci 111(13):1813–1821

Gimona M, Djinovic-Carugo K, Kranewitter WJ, Winder SJ (2002) Functional plasticity of CH domains. FEBS Lett 513:98–106

Godlewski J, Wang S, Wilson TG (2006) Interaction of bHLH-PAS proteins involved in juvenile hormone reception in Drosophila. Biochem Biophys Res Commun 342:1305–1311

Grad I, Niedziela-Majka A, Kochman M, Ozyhar A (2001) Analysis of Usp DNA binding domain targeting reveals critical determinants of the ecdysone receptor complex interaction with the response element. Eur J Biochem/FEBS 268:3751–3758

Greb-Markiewicz B, Orlowski M, Dobrucki J, Ozyhar A (2011) Sequences that direct subcellular traffic of the Drosophila methoprene-tolerant protein (MET) are located predominantly in the PAS domains. Mol Cell Endocrinol 345:16–26

Guo E, He Q, Liu S, Tian L, Sheng Z, Peng Q, Guan J, Shi M, Li K, Gilbert LI, Wang J, Cao Y, Li S (2012) MET is required for the maximal action of 20-hydroxyecdysone during Bombyx metamorphosis. PloS ONE 7:e53256

Hartwig JH (1995) Actin-binding proteins. 1: Spectrin super family. Prot Profile 2:703–800

Heyduk T, Heyduk E (2002) Molecular beacons for detecting DNA binding proteins. Nat Biotechnol 20:171–176

Heyduk E, Heyduk T, Claus P, Wisniewski JR (1997) Conformational changes of DNA induced by binding of Chironomus high mobility group protein 1a (cHMG1a). Regions flanking an HMG1 box domain do not influence the bend angle of the DNA. J Biol Chem 272:19763–19770

Heyduk E, Knoll E, Heyduk T (2003) Molecular beacons for detecting DNA binding proteins: mechanism of action. Anal Biochem 316:1–10

Himukai R, Kuzuhara T, Horikoshi M (1999) Relationship between the subcellular localization and structures of catalytic domains of FKBP-type PPIases. J Biochem 126:879–888

Hu X, Cherbas L, Cherbas P (2003) Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. Mol Endocrinol 17:716–731

Iakoucheva LM, Radivojac P, Brown CJ, O'Connor TR, Sikes JG, Obradovic Z, Dunker AK (2004) The importance of intrinsic disorder for protein phosphorylation. Nucleic Acids Res 32:1037–1049

Iwema T, Billas IM, Beck Y, Bonneton F, Nierengarten H, Chaumot A, Richards G, Laudet V, Moras D (2007) Structural and functional characterization of a novel type of ligand-independent RXR-USP receptor. EMBO J 26:3770–3782

Iwema T, Chaumot A, Studer RA, Robinson-Rechavi M, Billas IM, Moras D, Laudet V, Bonneton F (2009) Structural and evolutionary innovation of the heterodimerization interface between USP and the ecdysone receptor ECR in insects. Mol Biol Evol 26(4):753–68. doi: 10.1093/molbev/msn302. Epub 2009 Jan 6

Jakób M, Kołodziejczyk R, Orłowski M, Krzywda S, Kowalska A, Dutko-Gwóźdź J, Gwóźdź T, Kochman M, Jaskólski M, Ożyhar A (2007) Novel DNA-binding element within the C-terminal extension of the nuclear receptor DNA-binding domain. Nucleic Acids Res 35:2705–2718

Jindra M, Palli SR, Riddiford LM (2013) The juvenile hormone signaling pathway in insect development. Annu Rev Entomol 58:181–204

Jones G, Sharp PA (1997) Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. Proc Natl Acad Sci United States Am 94:13499–13503

Jones G, Wozniak M, Chu Y, Dhar S, Jones D (2001) Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. Insect Biochem Mol Biol 32:33–49

Jones G, Jones D, Teal P, Sapa A, Wozniak M (2006) The retinoid-X receptor ortholog, ultraspiracle, binds with nanomolar affinity to an endogenous morphogenetic ligand. FEBS J 273:4983–4996

Kapitskaya M, Wang S, Cress DE, Dhadialla TS, Raikhel AS (1996) The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. Mol Cell Endocrinol 121:119–132

Knoll E, Heyduk T (2004) Unimolecular beacons for the detection of DNA-binding proteins. Anal Chem 76:1156–1164

Konopova B, Jindra M (2007) Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle Tribolium castaneum. Proc Natl Acad Sci USA 104:10488–10493

Kozłowska M, Tarczewska A, Jakób M, Szpotkowski K, Wojtas M, Rymarczyk G, Ożyhar A (2014) Calponin-like chd64 is partly disordered. PLoS ONE 9:e96809

Krasowski MD, Reschly EJ, Ekins S (2008) Intrinsic disorder in nuclear hormone receptors. J Proteome Res 7:4359–4372

Krusiński T, Laskowska A, Ożyhar A, Dobryszycki P (2008) The application of an immobilized molecular beacon for the analysis of the DNA binding domains from the ecdysteroid receptor proteins Usp and EcR's interaction with the hsp27 response element. J Biomol Screen 13:899–905

Krusiński T, Ożyhar A, Dobryszycki P (2010) Dual FRET assay for detecting receptor protein interaction with DNA. Nucleic Acids Res 38:e108

Lane D, Prentki P, Chandler M (1992) Use of gel retardation to analyze protein-nucleic acid interactions. Microbiol Rev 56:509–528

Laudet VGH (2002) The nuclear receptors factsbook. Academic, London

Lehmann M, Korge G (1995) Ecdysone regulation of the Drosophila Sgs-4 gene is mediated by the synergistic action of ecdysone receptor and SEBP 3. EMBO J 14:716–726

Lehmann M, Wattler F, Korge G (1997) Two new regulatory elements controlling the Drosophila Sgs-3 gene are potential ecdysone receptor and fork head binding sites. Mech Dev 62:15–27

Li JJ, Fang X, Schuster SM, Tan W (2000) Molecular beacons: a novel approach to detect protein-DNA interactions. Angew Chem Int Ed Engl 39:1049–1052

Li Y, Zhang Z, Robinson GE, Palli SR (2007) Identification and characterization of a juvenile hormone response element and its binding proteins. J Biol Chem 282:37605–37617

Li ZQ, Cheng DJ, Wei L, Zhao P, Shu X, Tang L, Xiang ZH, Xia QY (2010) The silkworm homolog of Methoprene-tolerant (Met) gene reveals sequence conservation but function divergence. Insect Sci 17:313–324

Liu Y, Sheng Z, Liu H, Wen D, He Q, Wang S, Shao W, Jiang RJ, An S, Sun Y, Bendena WG, Wang J, Gilbert LI, Wilson TG, Song Q, Li S (2009) Juvenile hormone counteracts the bHLH-PAS

transcription factors MET and GCE to prevent caspase-dependent programmed cell death in Drosophila. Development (Cambridge England) 136:2015–2025
Liu PC, Wang JX, Song QS, Zhao XF (2011) The participation of calponin in the cross talk between 20-hydroxyecdysone and juvenile hormone signaling pathways by phosphorylation variation. *PloS one* 6:e19776
Malaney P, Pathak RR, Xue B, Uversky VN, Dave V (2013) Intrinsic disorder in PTEN and its interactome confers structural plasticity and functional versatility. Sci Rep 3:2035
Maletta M, Orlov I, Roblin P, Beck Y, Moras D, Billas IM, Klaholz BP (2014) The palindromic DNA-bound USP/EcR nuclear receptor adopts an asymmetric organization with allosteric domain positioning. Nat Commun 5:4139
McEwan IJ (2009) Nuclear receptors: one big family. Methods Mol Bio (Clifton NJ) 505:3–18
McGough A (1998) F-actin-binding proteins. Curr Opin Struct Bio 8:166–176
Miura K, Oda M, Makita S, Chinzei Y (2005) Characterization of the Drosophila Methoprene -tolerant gene product. Juvenile hormone binding and ligand-dependent gene regulation. FEBS J 272:1169–1178
Moore AW, Barbel S, Jan LY, Jan YN (2000) A genomewide survey of basic helix-loop-helix factors in Drosophila. Proc Natl Acad Sci USA 97:10436–10441
Mouillet JF, Henrich VC, Lezzi M, Vögtli M (2001) Differential control of gene activity by isoforms A, B1 and B2 of the Drosophila ecdysone receptor. Eur J Biochem 268:1811–1819
Nair RR, Solway J, Boyd DD (2006) Expression cloning identifies transgelin (SM22) as a novel repressor of 92-kDa type IV collagenase (MMP-9) expression. J Biol Chem 281:26424–26436
Nicolai M, Bouhin H, Quennedey B, Delachambre J (2000) Molecular cloning and expression of Tenebrio molitor ultraspiracle during metamorphosis and in vivo induction of its phosphorylation by 20-hydroxyecdysone. Insect Mol Bio 9:241–249
Niedziela-Majka A, Kochman M, Ozyhar A (2000) Polarity of the ecdysone receptor complex interaction with the palindromic response element from the hsp27 gene promoter. Eur J Biochem/FEBS 267:507–519
Nocula-Ługowska M, Rymarczyk G, Lisowski M, Ożyhar A (2009) Isoform-specific variation in the intrinsic disorder of the ecdysteroid receptor N-terminal domain. Proteins 76:291–308
Orłowski M, Szyszka M, Kowalska A, Grad I, Zoglowek A, Rymarczyk G, Dobryszycki P, Krowarsch D, Rastinejad F, Kochman M, Ożyhar A (2004) Plasticity of the ecdysone receptor DNA binding domain. Mol Endocrinol (Baltimore Md) 18:2166–2184
Ożyhar A, Pongs O (1993) Mutational analysis of the interaction between ecdysteroid receptor and its response element. J Steroid Biochem Mol Bio 46:135–145
Ożyhar A, Strangmann-Diekmann M, Kiltz HH, Pongs O (1991) Characterization of a specific ecdysteroid receptor-DNA complex reveals common properties for invertebrate and vertebrate hormone-receptor/DNA interactions. European J biochem/FEBS 200:329–335
Pakuła S, Orłowski M, Rymarczyk G, Krusiński T, Jakób M, Zoglowek A, Ożyhar A, Dobryszycki P (2012) Conformational changes in the DNA-binding domains of the ecdysteroid receptor during the formation of a complex with the hsp27 response element. J Biomol Struct Dyn 30:379–393
Perera SC, Zheng S, Feng QL, Krell PJ, Retnakaran A, Palli SR (2005) Heterodimerization of ecdysone receptor and ultraspiracle on symmetric and asymmetric response elements. Archives Insect Biochem Physiol 60:55–70
Petz LN, Nardulli AM, Kim J, Horwitz KB, Freedman LP, Shapiro DJ (1997) DNA bending is induced by binding of the glucocorticoid receptor DNA binding domain and progesterone receptors to their response element. J Steroid Biochem Mol Bio 60:31–41
Pieprzyk J, Zbela A, Jakób M, Ożyhar A, Orłowski M 2014. Homodimerization propensity of the intrinsically disordered N-terminal domain of Ultraspiracle from Aedes aegypti. Biochim Biophys Acta 1844:1153–1166
Potthoff SJ, Romine LE, Nardulli AM (1996) Effects of wild type and mutant estrogen receptors on DNA flexibility, DNA bending, and transcription activation. Mol Endocrinol (Baltimore Md) 10:1095–1106

Prendergast P, Pan Z, Edwards DP (1996) Progesterone receptor-induced bending of its target DNA: distinct effects of the A and B receptor forms. Mol Endocrinol (Baltimore Md) 10:393–407

Pursley S, Ashok M, Wilson TG (2000) Intracellular localization and tissue specificity of the Methoprene-tolerant (Met) gene product in Drosophila melanogaster. Insect Biochem Mol Bio 30:839–845

Rastinejad F (2001) Retinoid X receptor and its partners in the nuclear receptor family. Curr Opin Struct Bio 11:33–38

Rauch P, Grebe M, Elke C, Spindler KD, Spindler-Barth M (1998) Ecdysteroid receptor and ultraspiracle from Chironomus tentans (Insecta) are phosphoproteins and are regulated differently by molting hormone. Insect Biochem Mol Bio 28:265–275

Riddiford LM (1996) Juvenile hormone: the status of its "status quo" action. Archives Insect Biochem Physiol 32:271–286

Riddiford LM (2008) Juvenile hormone action: a 2007 perspective. J Insect Physiol 54:895–901

Riddihough G, Pelham HR (1986) Activation of the Drosophila hsp27 promoter by heat shock and by ecdysone involves independent and remote regulatory sequences. EMBO J 5:1653–1658

Riddihough G, Pelham HR (1987) An ecdysone response element in the Drosophila hsp27 promoter. EMBO J 6:3729–3734

Rymarczyk G, Grad I, Rusek A, Oświęcimska-Rusin K, Niedziela-Majka A, Kochman M, Ożyhar A (2003) Purification of Drosophila melanogaster ultraspiracle protein and analysis of its A/B region-dependent dimerization behavior in vitro. Biol Chem 384:59–69

Schmid FX (1993) Prolyl isomerase: enzymatic catalysis of slow protein-folding reactions. Annu Rev Biophys Biomol Struct 22:123–142

Seliga J, Bielska K, Wieczorek E, Orłowski M, Niedenthal R, Ożyhar A (2013) Multidomain sumoylation of the ecdysone receptor (EcR) from Drosophila melanogaster. J Steroid Biochem Mol Biol 138:162–173

Shemshedini L, Wilson TG (1990) Resistance to juvenile hormone and an insect growth regulator in Drosophila is associated with an altered cytosolic juvenile hormone-binding protein. Proc Natl Acad Sci USA 87:2072–2076

Shulemovich K, Dimaculangan DD, Katz D, Lazar MA (1995) DNA bending by thyroid hormone receptor: influence of half-site spacing and RXR. Nucleic Acids Res 23:811–818

Song Q, Gilbert LI (1998) Alterations in ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. Insect Biochem Mol Bio 28:849–860

Sun X, Song Q (2006) PKC-mediated USP phosphorylation is required for 20E-induced gene expression in the salivary glands of Drosophila melanogaster. Archives Insect Biochem Physiol 62:116–127

Sutherland JD, Kozlova T, Tzertzinis G, Kafatos FC (1995) Drosophila hormone receptor 38: a second partner for Drosophila USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. Proc Natl Acad Sci USA 92:7966–7970

Szamborska-Gbur A, Rymarczyk G, Orłowski M, Kuzynowski T, Jakób M, Dziedzic-Letka A, Górecki A, Dobryszycki P, Ożyhar A (2014) The molecular basis of conformational instability of the ecdysone receptor DNA binding domain studied by in silico and in vitro experiments. PloS ONE 9:e86052

Talbot WS, Swyryd EA, Hogness DS (1993) Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73:1323–1337

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tompa P (2002) Intrinsically unstructured proteins. Trends Biochem Sci 27:527–533

Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14:303–308

Uversky VN (2002) Natively unfolded proteins: a point where biology waits for physics. Prot Sci 11:739–756

Uversky VN (2013) The most important thing is the tail: multitudinous functionalities of intrinsically disordered protein termini. FEBS Lett 587:1891–1901

Uversky VN, Oldfield CJ, Dunker AK (2008) Intrinsically disordered proteins in human diseases: introducing the D2 concept. Annu Rev Biophys 37:215–246

Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J (1993) Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. J Mol Bio 229:105–124

Wang SF, Miura K, Miksicek RJ, Segraves WA, Raikhel AS (1998) DNA binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex. J Biol Chem 273:27531–27540

Wang S-F, Li C, Zhu J, Miura K, Miksicek RJ, Raikhel AS (2000) Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. Dev Bio 218:99–113

Wang S, Baumann A, Wilson TG (2007) Drosophila melanogaster Methoprene-tolerant (Met) gene homologs from three mosquito species: members of PAS transcriptional factor family. J Insect Physiol 53:246–253

Wang S, Wang J, Sun Y, Song Q, Li S (2012) PKC-mediated USP phosphorylation at Ser35 modulates 20-hydroxyecdysone signaling in Drosophila. J Proteome Res 11:6187–6196

Watanabe T, Takeuchi H, Kubo T (2010) Structural diversity and evolution of the N-terminal isoform-specific region of ecdysone receptor-A and -B1 isoforms in insects. BMC Evolut Biol 10:40-2148-10-40

Wilson TG, Ashok M (1998) Insecticide resistance resulting from an absence of target-site gene product. Proc Natil Acad Sci USA 95:14040–14044

Wilson TG, Fabian J (1986) A Drosophila melanogaster mutant resistant to a chemical analog of juvenile hormone. Dev Bio 118:190–201

Wilson TG, Yerushalmi Y, Donnell DM, Restifo LL (2006) Interaction between hormonal signaling pathways in Drosophila melanogaster as revealed by genetic interaction between methoprene-tolerant and broad-complex. Genetics 172:253–264

Wright PE, Dyson HJ (1999) Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. J Mol Biol 293:321–331

Xie H, Vucetic S, Iakoucheva LM, Oldfield CJ, Dunker AK, Obradovic Z, Uversky VN (2007) Functional anthology of intrinsic disorder. 3. Ligands, post-translational modifications, and diseases associated with intrinsically disordered proteins. J Proteome Res 6:1917–1932

Xu Y, Fang F, Chu Y, Jones D, Jones G (2002) Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle. Eur J Biochem/FEBS 269:6026–6036

Yang Z, Chang YJ, Miyamoto H, Ni J, Niu Y, Chen Z, Chen YL, Yao JL, Di Sant'agnese PA, Chang C (2007) Transgelin functions as a suppressor via inhibition of ARA54-enhanced androgen receptor transactivation and prostate cancer cell growth. Mol Endocrinol 21:343–358

Yao TP, Segraves WA, Oro AE, Mckeown M, Evans RM (1992) Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71:63–72

Yao TP, Forman BM, Jiang Z, Cherbas L, Chen JD, Mckeown M, Cherbas P, Evans RM (1993) Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature 366:476–479

Zhang Z, Xu J, Sheng Z, Sui Y, Palli SR (2011) Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. J Biol Chem 286:8437–8447

Zoglowek A, Orłowski M, Pakuła S, Dutko-Gwozdz J, Pajdzik D, Gwóźdź T, Rymarczyk G, Wieczorek E, Dobrucki J, Dobryszycki P, Ożyhar A (2012) The composite nature of the interaction between nuclear receptors EcR and DHR38. Biol Chem 393:457–471

Structural Analysis of Heterodimeric Nuclear Receptors

Brice Beinsteiner and Dino Moras

1 Introduction

Nuclear receptors share a common modular organization. A variable N-terminal domain harbors a ligand-independent activation function (AF-1), the conserved DNA binding domain (DBD), a connecting hinge and the C-terminal ligand binding domain (LBD) which contains the ligand dependent activation function AF-2 constitute the different modules. The DNA and the ligand binding domains (DBD and LBD) are structurally well characterized thanks to NMR and X-ray crystallography. Nearly 500 crystal structures of these domains in various functional states are deposited in the protein data bank (PDB). Remarkably the LBD fold first described in 1995 (Bourguet et al. 1995) has so far only been found in the superfamily of NRs and can be considered its signature structural motif. The two other domains (NTDs and hinges) are highly variable both in size and sequence and either intrinsically disordered (most NTDs) or with limited and variable secondary structures elements. These properties confer the necessary flexibility to adapt the NRs conformation to the different partners involved into the dynamics of the regulation process. Different conformations can potentially exist, the main question being what is the functional relevance of the observed structures?

Most NRs are known to function as dimers (homo or hetero) and with the exception of the group of oxosteroid receptors (AR, GR, MR, PR) all structural data point to a conserved interface in the LBDs dimer. A multiple alignment of NR LBD sequences revealed two sequence motifs that partition the superfamily into

D. Moras (✉) · B. Beinsteiner
Centre for Integrative Biology (CBI), Department of Integrated Structural Biology, Institute of Genetics and of Molecular and Cellular Biology (IGBMC), 1 rue Laurent Fries, Illkirch, France
e-mail: moras@igbmc.fr

Institut National de la Santé et de la Recherche Médicale (INSERM) U964, Illkirch, France

Centre National de la Recherche Scientifique (CNRS) UMR 7104, Illkirch, France

Université de Strasbourg, Strasbourg, France

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_7

two classes related to their oligomeric behavior (Brelivet et al. 2004). The class II subgroup encompasses all NRs known to function as heterodimers with RXR or ultraspiracle (USP). USP forms heterodimers with the ecdysone receptor (EcR) to regulate ecdysteroid-dependent molting and development. The class II specific salt bridges that connect H4/H5 to L8-9 and H8 to H9 suggest the existence of class-specific pathways that transfer allosteric signals through the LBD backbone. A first illustration of this concept was provided by the study of the role of phosphorylation in the activation process of RAR (Gaillard et al. 2006). Their partners (RXRs or USP) belong to class I and form stable homodimers in absence of class II NRs (Billas et al. 2001).

NRs interact with corepressors, coactivators and other protein cofactors that participate in signal transduction of the basal transcriptional machinery (Bulynko and O'Malley 2011). More than 300 primary or secondary cofactors have been identified (www.nursa.org), but their roles have not been fully elucidated. A general model proposes that nonsteroidal NRs, form heterodimers with RXRs. In the absence of ligand, the heterodimers are associated with corepressor complexes with histone-deacetylase activity that modify chromatin to establish and maintain a repressed transcriptional state (Nagy et al. 1999; Glass and Rosenfeld 2000). The binding of ligands induce structural transitions in the LBDs leading to the release of the corepressors and the formation of a novel interaction surface for coactivators, or components of the basal transcription machinery (Aoyagi and Archer 2008).

The structural studies of DNA bound full length or ΔAB receptors allow a better understanding of the structure-function relationships at the molecular level. Two crystal structures of heterodimers are presently known (PPARγ/RXRα/DNA and LXRβ/RXRα/DNA) (Chandra et al. 2008; Lou et al. 2014). Two EM structures of frozen solutions (cryo-EM) are also available for VDR/RXRα/DNA and USP/EcR/DNA (Orlov et al. 2012; Maletta et al. 2014). Several solution structures of full length heterodimers in their free state and/or bound to various DNA response elements and cofactors have been determined using integrative approaches (RARα-RXRα, VDR-RXRα, PPARγ-RXRα and USP/EcR) (Rochel et al. 2011; Osz et al. 2012; Maletta et al. 2014). One crystal structure and solutions structures of homodimers bound to their target DNAs are also available (Takacs et al. 2013; Chandra et al. 2013). The results illustrate the flexibility of the NRs while revealing common features for the molecular architecture of the complexes. Some functional correlations emerge.

2 Methodological Considerations

Solution structures were determined using integrative approaches that combine small angle diffraction methods by X-Rays (SAXS) and neutrons (SANS), optical techniques like FRET with labelled molecules and electron microscopy (cryo-EM). Note that the cryo-EM method used for the structure determination analyses a frozen solution that preserves the sample in a functional state. SAXS and SANS are powerful structural methods to study flexible multi-domain proteins in solution

avoiding crystal packing artifacts (Petoukhov et al. 2013). They provide molecular envelopes that can be interpreted at the molecular level when high resolution models of individual domains are available, which is the case for NRs. SANS allows masking part of the complex using variable D_2O/H_2O ratios for the solvent (the contrast variation method). These methods allow addressing unambiguously the question of the overall correct topology of a complex in solution. Furthermore they provide important additional information like the presence or not of a single or largely dominant conformer in solution and its correlation with proposed molecular models.

Crystallography provides high resolution information on the ordered part of the molecules but relies on the existence of good diffracting crystals. The crystal packing captures the conformation most favorable for crystal growth and thus the crystal structure provides a snapshot of a selected conformer. The latter may only represent a small fraction of the solution conformers since the crystallization process can trap and stabilize a conformation not dominant in solution. As a matter of fact, cases have been reported in the literature where a contaminant, present at less than 5 % of the protein preparation, was crystallized (Veesler et al. 2008).

3 DNA Binding

The first crystal structures of DNA bound DBDs reported were those of the GR and ER homodimers on their cognate symmetric repeats (Luisi et al. 1991; Schwabe et al. 1993) . Additional crystal structures with the DBD of RXR in various combinatorial partnerships on direct repeats followed (Rastinejad et al. 1995, 2000; Zhao et al. 2000). These and most other crystal structures like those of full length receptors have been obtained with consensus DNA sequences, extremely rare in real functional sequences of target promoters. The first structural evidence of the importance of the real sequence was provided by the crystal structure of USP/EcR DBDs bound to half-sites with a 1 base-pair spaced inverted repeat (IR1), a natural pseudopalindromic DNA response element (RE) reminiscent of IRs observed for vertebrate steroid hormone receptors (Jakób et al. 2007). Comparison of the structure with that obtained using an idealized response element, showed how the EcR accommodates DNA-induced structural changes. Part of the C-terminal extension (CTE) of the EcR DBD folds into an α-helix whose location in the minor groove did not match any of the previously observed locations. Mutational analyses suggest that the α-helix is indispensable for DNA-binding. In 2009 a structure-function relationships study showed that the sequence of the GR binding sites differentially affects receptor conformation and transcriptional activity (Meijsing et al. 2009). Although only minor structural changes could be observed when comparing the crystal structures of DBDs bound to different response elements, a correlation with GR activity supports the proposal of DNA being an allosteric effector to modulate the receptor activity.

ChIP-chip and ChIP-seq experiments in different cell types have pointed to thousands of potential genomic RAR binding sites and RA-regulated genes networks

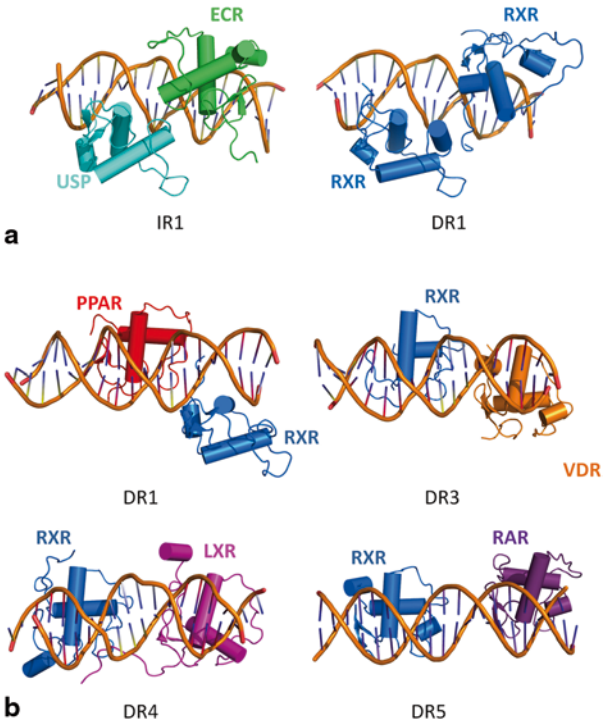


Fig. 1 **a** *Left*: DNA Binding Domains (*DBD*) of the heterodimer *USP*/*EcR* on the DNA target, an inverted repeat separated by one base (*IR1*). PDB ID: 2HAN. *Right*: DBDs of homodimer *RXR*. The DNA target is a direct repeat separated by one base (*DR1*) PDB ID: 4CN2. **b** DNA Binding Domains (*DBD*) on direct repeat separated by 1, 3, 4 and 5 bases (*DR1*, *DR3*, *DR4* and *DR5*). PDB ID: 3DZU for *PPAR*/*RXR* DBD *DR1*. CryoEM structure for *VDR*/*RXR* DBD *DR3*. PDB: ID 4NQA for *LXR*/*RXR* DBD *DR4*. SAXS/SANS/FRET structure for *RAR*/*RXR* DBD *DR5*

(Hua et al. 2009; Moutier et al. 2012). Analysis of the RAR bound loci revealed the presence of the classical direct repeat (DR) elements with 1, 2 and 5 base pair spacers and numerous non-canonical sites. RARα/RXRα heterodimers were shown to bind to the asymmetric DRs with specific polarities (Perlmann et al. 1993; Mader et al. 1993). In the heterodimers bound to DR5 the DBD of RAR binds to the half-site at the 3′ end of the DR while RXR binds to the 5′ half-site. The polarity is reversed in the case of DR1 with RXR bound to the 3′half-site. The recent crystallographic study of RXR DBDs on DR1 response elements explains how the 3′ end of the response element is selected (Osz et al. 2015) (Fig. 1). It also shows that natural DR1s are bound with higher affinity than an idealized symmetric DR1. Subtle changes in the consensus DR1 DNA sequence specify binding affinity through altered RXR-DBD-DNA contacts and changes in DBD conformation.

4 LBDs Heterodimers

The LBD fold is constituted by a primarily helical scaffold termed “antiparallel α-helical sandwich” of 12 helices and a short β-turn arranged in three layers. This arrangement generates a mostly hydrophobic ligand-binding pocket (LBP) which can accommodate natural or synthetic ligands. The pocket can adapt to accommodate

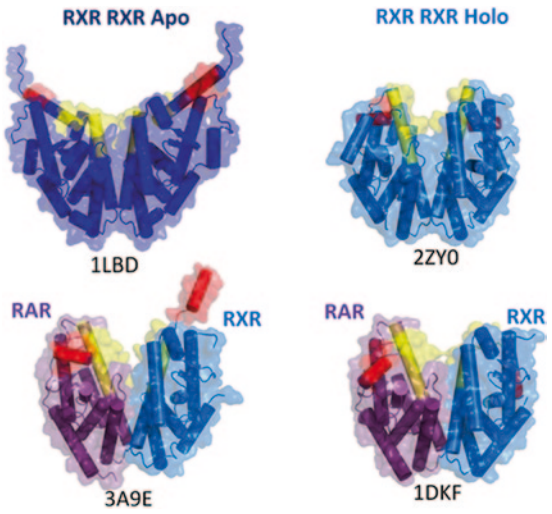


Fig. 2 Dimers of Ligand Binding Domains (*LBD*), the helices H11 and H12 are colored in *yellow* and *red* respectively. *Top*: *left* homodimer of apo-*RXR*s (without ligand) (*1LBD*), *right*: homodimer of liganded *RXR*s in the agonist conformation (*2ZY0*). *Bottom*: heterodimers of liganded *RAR*/*RXR*, *RAR* is bound to agonist ligands, whereas *RXR* is bound to an antagonist (*3A9E*, *left*) or an agonist ligand (*1DKF*, *right*)

different ligands (Billas et al. 2003; Huet et al. 2015). In the agonist-bound (holo) LBD the C-terminal helix H12 is stabilized against H3 and H11, thereby sealing the ligand-binding cavity. This conformation is specifically induced by the binding of agonists and is referred to as the "active conformation". It favors the recruitment of transcriptional coactivators to the receptor surface composed of helices H3, H4 and H12.

The crystal structures of a limited number of unliganded (apo) LBDs (Bourguet et al. 1995), together with biochemical data, have revealed that H12 either adopts a different position or exists as a dynamic ensemble of conformations (Nahoum et al. 2007; le Maire et al. 2010). In both apo-RXR from human (hRXR) and the invertebrate chordate amphioxus (AmphiRXR), the LBPs are filled with hydrophobic residues of H11, thereby stabilizing the ligand-free cavity. Comparison of the apo- and holo-LBD structures of hRXRα illustrates the mechanism by which the activation function AF-2 becomes transcriptionally competent upon ligand binding (Egea et al. 2000, 2001) (Fig. 2). The structural transition essentially involves the stabilization of helix H11 in the continuity of helix H10, and the repositioning of helix H12 that seals the LBP and further stabilizes ligand binding.

Several structures of homo- and hetero-dimers, notably those of RXR LBD in complex with various partner LBDs, including RAR (Bourguet et al. 2000; Pogenberg et al. 2005; Sato et al. 2010), the peroxisome proliferator activated receptor (Gampe et al. 2000), the thyroid hormone receptor (Putcha et al. 2012), the liver X receptor (Svensson et al. 2003) or the constitutive androstane receptor (Suino et al. 2004; Xu et al. 2004), have been reported thereby identifying the structural organization of receptor dimers. All these structures demonstrate a topologically conserved dimerization surface with residues from helices H7, H9, H10, as well as loops L8–9 and L9–10 of each protomer forming a network of complementary hydrophobic and charged residues. Upon dimerization the hidden surface of each monomer is about 1000 $Å^2$ (Table 1). Extensive analyses of the dimerization inter-

Table 1 Buried surface of representative homo and hetero dimers. (Binding area of 2 monomers)

PDB	Dimer		Surface dimer (A²)	Buried surface (A²)
	Chain A	Chain B	Complex	Complex
1LBD	RXRα	RXRα	23073,543	1836,076
2ZY0	RXRα	RXRα	21496,027	2187,918
3A9E	RXRα	RARα	24607,131	1600,08
1DKF	RXRα	RARα	21676,182	1881,687

faces based on crystal structure and sequence alignment have provided insights for the specific dimerization characteristics of nuclear receptors (Bourguet et al. 2000; Brelivet et al. 2004).

5 DNA Binding Mechanism and Architecture of the Complexes

In solution RAR and RXR form stable elongated complexes with bound LBD heterodimers and DBDs loosely positioned through their flexible hinge domains (Fig. 3). Upon binding to target DNA, the heterodimer adopts an asymmetric conformation with two distinct modules, the DBDs bound to the DNA RE and the heterodimeric LBDs, positioned at the 5-end of the RE. The structures then suggest that DNA recognition is achieved by the preassembled heterodimer instead of the heterodimer being constituted on the DNA target. The process of a combined approach to the recognition of the response element is an efficient way to overcome the specificity problem with low affinity constants for each single DBD. It is tempting to generalize the mechanism to most NRs but more experimental data are needed.

The structural data explain how DNA dictates the architecture of the complex and its asymmetry, and several pieces of evidence further support the proposal. Velasco et al. showed that the binding of the SRC-1 receptor interacting domain to thyroid hormone receptor (TR) is influenced by the DNA response elements (Velas-

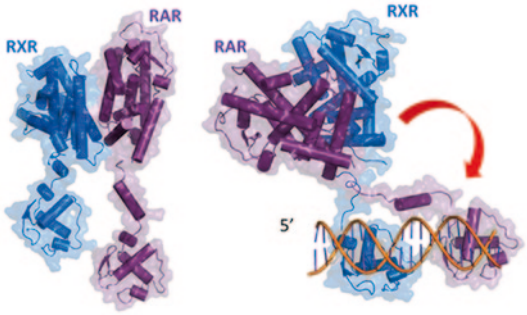


Fig. 3 Two solution structures of full length *RAR*/*RXR* in their free state (*left*) and bound to their target *DNA* (*DR5*) (*right*)

co et al. 2007). The thyroid response element organization dictates the composition of active receptor. An allosteric communication between SRC-1 and DNA was also reported for VDR-RXR (Zhang et al. 2011).

The solution structures of full length or ΔAB RAR/RXR heterodimers free and bound to the best characterised RAREs (DR1 and DR5) together with that of PPARα/RXRα/DR1 and VDR/RXRα on its DR3 response element were first determined using integrative approaches (Rochel et al. 2011). The molecular structures revealed some common features that suggest functional correlations that could be relevant for a large fraction of the superfamily. Regardless of the different positions and polarities of the bound DBDs the complexes exhibit a similar extended and asymmetric conformation with the LBD dimer positioned on the 5′ side of the response elements. In all cases the LBDs and the DBDs/DNA entities are nearly orthogonal, the pseudo two-fold symmetry axis of the LBDs and the DNA forming the two branches of an L-shaped structure. The LBDs dimers can rotate around both their pseudo two-fold axis and the DNA axis under the control of the flexible hinge domains. The position of the DBDs on the DNA response elements is dictated by the relative position of the binding motifs. For example addition of one base pair to the spacer sequence induces a shift of approximately 3.5 Å and a rotation of 36°. Thus for a given heterodimer the response elements control the architecture of the complexes through the polarity of the hexa-nucleotide binding motifs and the number of dinucleotide spacers. Together with the hinges they modulate the rotation of the LBDs and the relative position of the receptors. The hinge domains play an important role (see below).

The cryo-EM structure of the liganded human RXR and VDR bound to a consensus DNA response element forming a direct repeat (DR3) fits the experimental SAXS data and confirms the features of the solution structure (Orlov et al. 2012). The LBDs are perpendicular to the DNA and are located asymmetrically at the DNA 5′ end of the response element. The hinges of both VDR and RXR are fully visible. They hold the complex in an open conformation. The asymmetric topology of the complex provides the structural basis for RXR being an adaptive partner within NR heterodimers while the helical structure of VDR's hinge connects the 3′-bound DBD with the 5′ bound LBD in a more constrained and specific manner (see below).

The cryo EM structure of the USP/EcR/IR1 complex provides the first description of a full length receptor bound to an inverted repeat (Fig. 4). USP/EcR binds to half-sites with a 1 base-pair spaced inverted repeat (IR1), a palindromic DNA response element reminiscent of IRs observed for vertebrate steroid hormone receptors (SHRs). The structure reveals that even though the DNA is almost symmetric, the complex adopts a highly asymmetric architecture in which the ligand-binding domains (LBDs) are positioned 5′ off-centered on the RE which leads to additional interactions between the USP LBD and the 5′-flanking sequence that trigger transcription activity as monitored by transfection assays. With respect to an orientation perpendicular to the DNA, the LBDs are slightly tilted (~20°) towards the 5′ direction and rotated by ~30° around the pseudo-two-fold symmetry axis that goes through the LBD interface of USP and EcR. This results in an asymmetric arrangement where the USP LBD is closer to the DNA than the EcR LBD which leads to

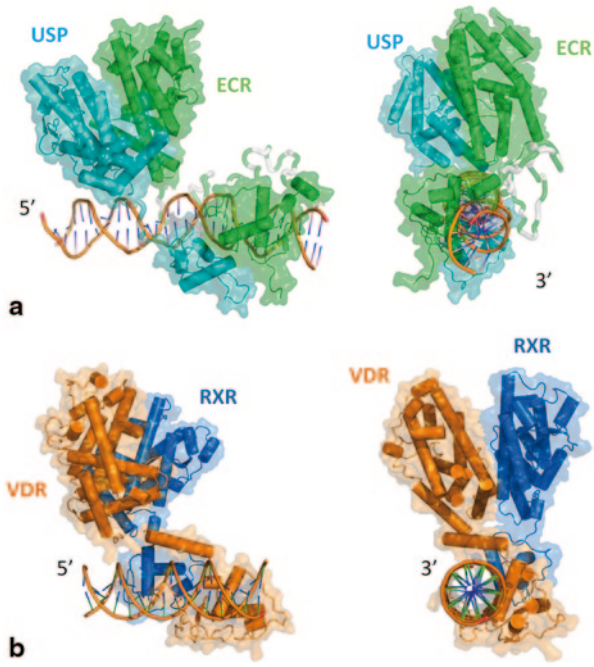


Fig. 4 **a** CryoEM structure of *EcR*/*USP* bound to the target DNA *IR1* (inverted repeat separated by 1 bases). The hinge part is modeled (not present in the PDB: 4UMM). **b** Two orthogonal views of the cryo-EM structure of *VDR*/*RXR* bound to their target DNA (DR3)

additional interactions between the USP LBD and the 5′-flanking sequence that trigger transcription activity as monitored by transfection assays. Although the LBDs of USP and EcR are pseudo-symmetric they can be distinguished unambiguously in the cryo-EM map thanks to specific structural differences. Remarkably, compared to DR-bound NR complexes, the position of the LBDs is inverted in the IR1-bound complex, a topology which arises from the opposite orientation of the second RE half-site. The structure shows that helix H12 of EcR it is in the agonist position (due to presence of the ecdysteroid ligand) while that of USP adopts an antagonist conformation due to the presence of a phospholipid ligand (Billas et al. 2003).

6 The Hinge Domains

The architecture of the different complexes points to the important role of the hinge domains in establishing and/or maintaining the integrity of the functional structures. The Table 2 illustrates the large size fluctuation of these domains. The flexibility of the RXR hinge is a structural necessity to adapt to numerous partners and even more response elements with different polarities and spacers. Conversely the hinges of the partner receptors may have a more constrained structure in order to improve the specificity of DNA recognition while stabilizing the functional architecture. VDR and the thyroid hormone receptor (TR) provide a good example of these properties.

Table 2 Hinge domain size

NR	Number of residues	Average
VDR	33	33
TR	38	38
RAR	25–26	25,11
LXR	41–63	50,75
EcR	71–93	79,57
FXR	51–56	54,28
PPAR	30–31	30,47
HNF4	17–20	17,37
RXR	20–29	22,05
USP	20–61	31

The structure of VDR/RXRα/DR3 shows that the long and extended hinge domain of RXR is needed to reach helix H1 on the side opposite from the RXR DBD. For RXR a rigid C terminal helix similar to that of VDR positioned at the 5′ side of the response element would entirely change the topology of the complex.

In the VDR/RXRα/DNA complex the helix orientation fits both the experimental molecular envelope and the distance requirements to join the N terminus of the LBD. A close, compact conformation for RXR–VDR would require the disruption of the helix. Mutation studies on VDR and RXR hinges (14 deletion mutants for VDR and 8 deletion mutants for RXR) fully support the solution structure, notably the requirement of the entire VDR hinge for full transcriptional activation (Shaffer et al. 2005). Deletion of region 114–120 is deleterious, but the mutation of the residues into alanine does not affect transcription (Hsieh et al. 1999). The role of this sequence-independent region is structural: it controls the relative spatial positions of the LBD and the DBD. By contrast, the mutation of residues 108–114 into alanine leads to a loss of transcription capability, underlying the critical role of these residues in positioning the LBD and DBD through contacts with the promoter. On the other hand up to 14 residues can be deleted from the more flexible hinge domain of RXR without marked effect on the transcription activity. This flexibility confers RXR plasticity and adaptability towards different response elements and heterodimeric partners. Interestingly in the crystal structures of DNA bound DBDs the α-helical hinge domains of VDR and TR exhibit similar orientations despite different crystal packing (Rastinejad et al. 1995; Shaffer and Gewirth 2002, 2004). The sequence homology between VDR and TR hinges suggest a molecular model of TR/RXR/DR4 fitting all previously defined functional criteria (Fig. 5)

7 Cofactors Binding and Stoichiometry

An asymmetric structure of the NRs-DNA complex favors the discrimination and long distance recognition of each monomer. The molecular structures show how the asymmetric positions of the LBDs onto the response elements generate different

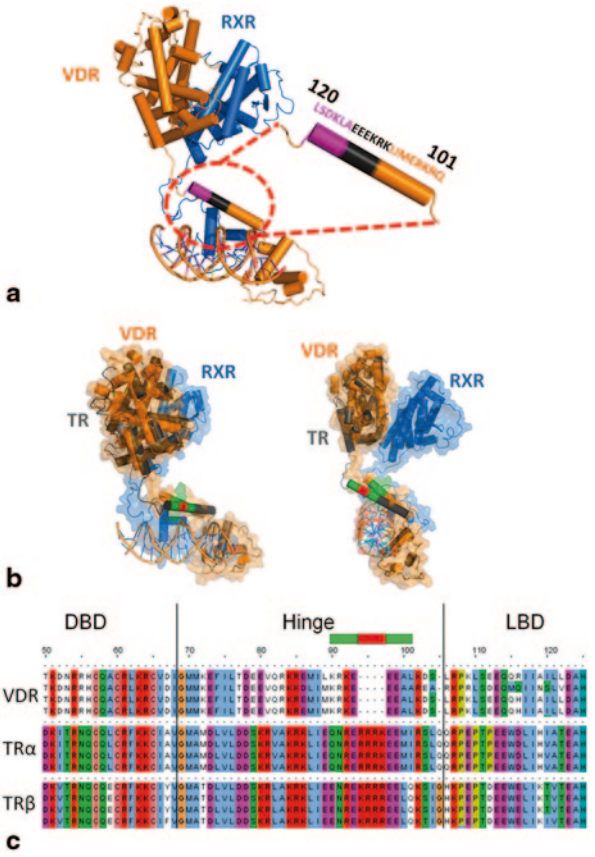


Fig. 5 **a** Solution structure of *VDR*/*RXR*/DNA. The zoomed area is the hinge part of *VDR*. The entire hinge is required for full transcriptional activity. In *magenta*? (114–120) it's a sequence independent spacer. In *black* (108–114) it's a sequence where mutations to alanine leads to a loss of transcriptional activity. **b** Two orthogonal views of the molecular models of *VDR*/*RXR*/DR3 (solution structure) and *TR*/*RXR*/DR4 (proposed model) superimposed. *VDR* is in *orange* and *TR* in *grey*. The larger hinge of *TR* allows to reach a *LBD* in a position close to that of *VDR* despite a longer distance (DR4). For *TR* model the crystal structures of *DBD* (PDB ID: 3M9E) and *LBD* (PDB ID: 3UVV) were used. For the hinge model the homologous amino acids between *VDR* and *TR* are shown in *green*, the insertion in the *TR* sequence is in *red*. **c** Sequence alignment of *VDR* and *TR* hinges. The *green* and *red* parts are correlated with the Fig. 5b

potential interfaces combining DNA and protein accessible surfaces. Several structural models have been proposed for the binding of coactivators to the LBDs. Based on the finding that the primary sequence of the cofactors binding domain usually exhibit two or three LXXLL binding motifs, it was postulated that either two cofactors could bind to one heterodimer or only one to both receptor using two motifs (the hat model). In the numerous crystal structures of LBD dimers in complex with short cofactor peptides the stoichiometry is always 2/2 supporting both models.

The solution studies of DNA complexes with full-length receptors RARα/RXRα and VDR/RXRα bound to DNA provided the first unambiguous structural evidence for a 2/1 stoichiometry for the receptors/coactivator complex (Rochel et al. 2011). Each heterodimer binds only one Med1 or SRC-1 coactivator protein via the partner of RXR, this preferential binding being controlled by affinity, rather than steric exclusion (Fig. 6). Indeed mutations of residues in the coactivator binding cleft of RXR do not affect the stoichiometry while RAR's antagonists prevent coactivator binding. The spatial positioning of the bound cofactor is thus imposed by the architecture of the complex and its selective affinity for the RXR partner. The molecular

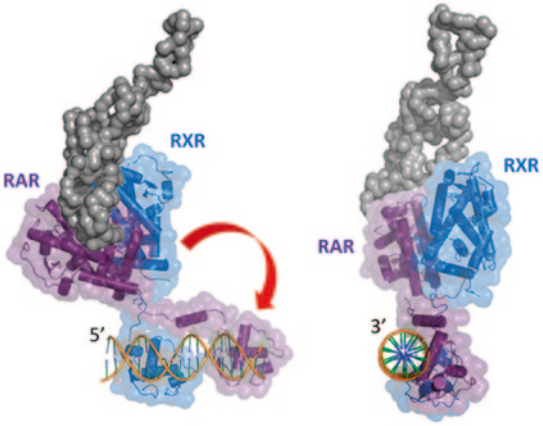


Fig. 6 Two orthogonal views of a coactivator domain (Med-1) in *grey* bound to *RAR*/*RXR*/DR5. The structure is based on experimental SAXS data

model of RXR/RAR on the RARβ2 promoter illustrates the functional correlation. The SRC-1 or Med1 receptor interacting domain is on one side of the DNA opposite to the RXR LBD and DBDs. In this position, the bound p160 coactivator is ideally positioned to reach its protein targets, the histone tails of H3 and H4.

Numerous data support the general character of the results, namely the asymmetric binding of only one coactivator interacting domain on both heterodimers and homodimers LBDs or full length receptors (Takacs et al. 2013; Fattori et al. 2014). The different stoichiometry observed using short peptides can be explained by the large excess of peptides in the crystallization batches together with a lack of steric hindrance due to the size of the peptide. The high concentration of peptides compensate for the lower affinity for the second binding site. When comparing the electron densities of the two bound peptides the observed asymmetric level is in agreement with different affinities (Osz et al. 2012).

8 Crystal Structures

Two out of the three crystal structures ((HNF4)2/DNA and LXRα/RXRα/DNA) exhibit an asymmetric open conformation similar to that observed in solution studies (Fig. 7). The LXR complex has an X-shaped arrangement, with DNA- and ligand-binding domains crossed, in contrast to the parallel domain arrangement of other NRs. Except for the conformation of the antagonist bound PPAR LBD, the high resolution crystal structures of full length NRs are consistent with previous results obtained with isolated DBDs/DNA complexes and LBDs. These results support the choice of the latter for the molecular modelling based on the low resolution envelops provided by the solution studies. All crystal structures show that DNA binding involves canonical contacts and auxiliary contacts that enhance affinity for the response element**.** The flexible hinges are only partially ordered and the mostly intrinsically disordered N terminal domains are not visible.

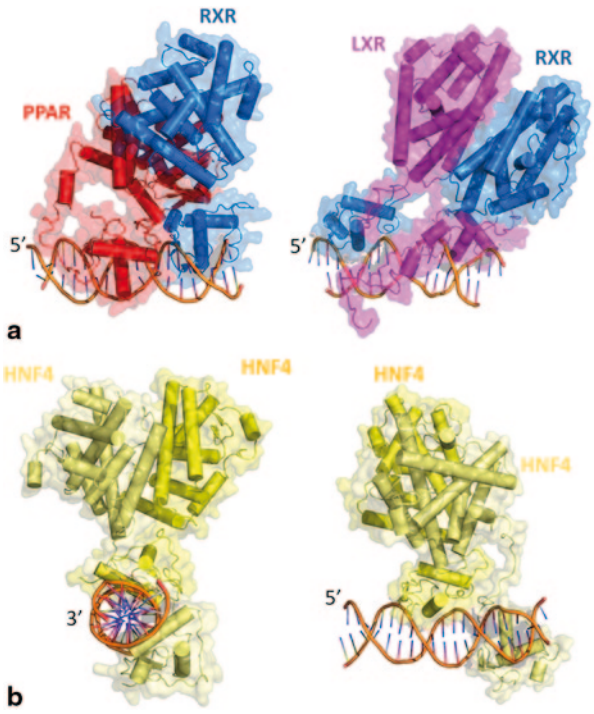


Fig. 7 Crystal structures. **a** *Left*: the compact heterodimer PPAR/RXR/DR1 (PDB ID: 3DZU). *Right*: the heterodimer LXR/RXR/DR4 exhibits an asymmetric open conformation (PDB ID: 4NQA). **b** Two views of the homodimer HNF4 bound to its target DNA (DR1) (PDB ID: 4IQR)

9 PPAR/RXR/DNA

The compact model of PPARγ/RXRα/DNA does not fit the experimental SAXS data (Rochel et al. 2011). The discrepancy between the calculated radius of gyration of the compact model (35 Å) and the experimental value (50 Å) suggests that the crystal structure represents only a minor part of the solution conformers. Furthermore the functional relevance of the original contact between PPAR LBD and RXR DBD is supported by a single mutation (Phe347Ala in the PPAR LBD). The closest RXR residue being at 6 Å of that phenylalanine, the mutation most likely affects the close ligand binding pocket and consequently the transcriptional activity of the complex. Further the position of Ser245 (Ser273 in PPARγ1) at the LBD/DBD interface is not consistent with a phosphorylation event, for which accessibility to the kinase would be required (Choi et al. 2010).

The agonist conformations for the antagonist bound PPAR LBDs contrast with the observations in high resolution X-ray structures of related LBD complexes (Xu et al. 2002). Crystal packing artifacts can explain the observation since the functionally important helix 12 makes a critical packing contact with DNA. A small fraction of agonist conformers in equilibrium with the antagonist ones could be present and selected but the electron density map and the conformation of the surrounding pocket residues cast serious doubts on the presence of the antagonist ligand GW9662.

10 Conclusion

The molecular structures of nuclear receptors bound to different response elements encompassing direct or inverted repeats reveal a few main common features: (i) the L-shape open conformation of the complexes (ii) the asymmetric position of LBDs at the 5′ end of the target DNAs for all complexes but LXRβ/RXRα (iii) the binding of only one coactivator molecule to the heterodimer, the partner of RXR being the anchoring module. These features rationalize the key role of DNA and the importance of the flanking sequences. The response elements direct the position of the LBDs on the DNA helix, which in turn fix the position of the cofactors binding site.

The functional relevance of the observed architectures and our understanding of the dynamics of transcription regulation by NRs will require more structural information from complexes with different coactivators and corepressors (full proteins or at least interacting domains). For this step EM and integrative approaches are the best suited and will be necessary.

References

Aoyagi S, Archer TK (2008) Dynamics of coactivator recruitment and chromatin modifications during nuclear receptor mediated transcription. Mol Cell Endocrinol 280:1–5. doi:10.1016/j.mce.2007.08.016

Billas IM, Moulinier L, Rochel N, Moras D (2001) Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. J Biol Chem 276:7465–7474. doi:10.1074/jbc.M008926200

Billas IML, Iwema T, Garnier J-M et al (2003) Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. Nature 426:91–96. doi:10.1038/nature02112

Bourguet W, Ruff M, Chambon P et al (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature 375:377–382. doi:10.1038/375377a0

Bourguet W, Germain P, Gronemeyer H (2000) Nuclear receptor ligand-binding domains: Three-dimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol Sci 21:381–388. doi:10.1016/S0165-6147(00)01548-0

Brelivet Y, Kammerer S, Rochel N et al (2004) Signature of the oligomeric behaviour of nuclear receptors at the sequence and structural level. EMBO Rep 5:423–429. doi:10.1038/sj.embor.7400119

Bulynko YA, O'Malley BW (2011) Nuclear receptor coactivators: Structural and functional biochemistry. Biochemistry (Mosc) 50:313–328. doi:10.1021/bi101762x

Chandra V, Huang P, Hamuro Y et al (2008) Structure of the intact PPAR-g-RXR-a nuclear receptor complex on DNA. Nature 456:350–356. doi:10.1038/nature07413

Chandra V, Huang P, Potluri N et al (2013) Multidomain integration in the structure of the HNF-4a nuclear receptor complex. Nature 495:394–398. doi:10.1038/nature11966

Choi JH, Banks AS, Estall JL et al (2010) Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature 466:451–456. doi:10.1038/nature09291

Egea PF, Mitschler A, Rochel N et al (2000) Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. EMBO J 19:2592–2601. doi:10.1093/emboj/19.11.2592

Egea PF, Rochel N, Birck C et al (2001) Effects of ligand binding on the association properties and conformation in solution of retinoic acid receptors RXR and RAR. J Mol Biol 307:557–576. doi:10.1006/jmbi.2000.4409

Fattori J, Campos JLO, Doratioto TR et al (2014) RXR agonist modulates TR: Corepressor dissociation upon 9-cis retinoic acid treatment. Mol Endocrinol (Baltim Md me20141251). doi:10.1210/me.2014–1251

Gaillard E, Bruck N, Brelivet Y et al (2006) Phosphorylation by PKA potentiates retinoic acid receptor alpha activity by means of increasing interaction with and phosphorylation by cyclin H/cdk7. Proc Natl Acad Sci U S A 103:9548–9553. doi:10.1073/pnas.0509717103

Gampe RT, Montana VG, Lambert MH et al (2000) Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. Mol Cell 5:545–555

Glass CK, Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14:121–141

Hsieh JC, Whitfield GK, Oza AK et al (1999) Characterization of unique DNA-binding and transcriptional-activation functions in the carboxyl-terminal extension of the zinc finger region in the human vitamin D receptor. Biochemistry (Mosc) 38:16347–16358

Hua S, Kittler R, White KP (2009) Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. Cell 137:1259–1271. doi: 10.1016/j.cell.2009.04.043

Huet T, Laverny G, Ciesielski F et al (2015) A vitamin D receptor selectively activated by gemini analogs reveals ligand dependent and independent effects. Cell Rep doi:10.1016/j.celrep.2014.12.045

Jakób M, Kołodziejczyk R, Orłowski M et al (2007) Novel DNA-binding element within the C-terminal extension of the nuclear receptor DNA-binding domain. Nucleic Acids Res 35:2705–2718. doi:10.1093/nar/gkm162

Maire AL, Teyssier C, Erb C et al (2010) A unique secondary-structure switch controls constitutive gene repression by retinoic acid receptor. Nat Struct Mol Biol 17:801–807. doi:10.1038/nsmb.1855

Lou X, Toresson G, Benod C et al (2014) Structure of the retinoid X receptor a–liver X receptor b (RXRa–LXRb) heterodimer on DNA. Nat Struct Mol Biol 21:277–281. doi:10.1038/nsmb.2778

Luisi BF, Xu WX, Otwinowski Z et al (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505. doi:10.1038/352497a0

Mader S, Chen JY, Chen Z et al (1993) The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificites of the DNA binding domains. EMBO J 12:5029–5041

Maletta M, Orlov I, Roblin P et al (2014) The palindromic DNA-bound USP/EcR nuclear receptor adopts an asymmetric organization with allosteric domain positioning. Nat Commun doi:10.1038/ncomms5139

Meijsing SH, Pufall MA, So AY et al (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324:407–410. doi:10.1126/science.1164265

Moutier E, Ye T, Choukrallah M-A et al (2012) Retinoic acid receptors recognize the mouse genome through binding elements with diverse spacing and topology. J Biol Chem 287:26328–26341. doi:10.1074/jbc.M112.361790

Nagy L, Kao HY, Love JD et al (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev 13:3209–3216

Nahoum V, Pérez E, Germain P et al (2007) Modulators of the structural dynamics of the retinoid X receptor to reveal receptor function. Proc Natl Acad Sci U S A 104:17323–17328. doi:10.1073/pnas.0705356104

Orlov I, Rochel N, Moras D, Klaholz BP (2012) Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. EMBO J 31:291–300. doi:10.1038/emboj.2011.445

Osz J, Brélivet Y, Peluso-Iltis C et al (2012) Structural basis for a molecular allosteric control mechanism of cofactor binding to nuclear receptors. Proc Natl Acad Sci U S A 109:E588–E594. doi:10.1073/pnas.1118192109

Osz J, McEwen AG, Poussin-Courmontagne P et al (2015) Structural basis of natural promoter recognition by the retinoid x nuclear receptor. Sci Rep 5:8216. doi:10.1038/srep08216

Perlmann T, Rangarajan PN, Umesono K, Evans RM (1993) Determinants for selective RAR and TR recognition of direct repeat HREs. Genes Dev 7:1411–1422

Petoukhov MV, Billas IML, Takacs M et al (2013) Reconstruction of quaternary structure from X-ray scattering by equilibrium mixtures of biological macromolecules. Biochemistry (Mosc) 52:6844–6855. doi:10.1021/bi400731u

Pogenberg V, Guichou J-F, Vivat-Hannah V et al (2005) Characterization of the interaction between retinoic acid receptor/retinoid X receptor (RAR/RXR) heterodimers and transcriptional coactivators through structural and fluorescence anisotropy studies. J Biol Chem 280:1625–1633. doi:10.1074/jbc.M409302200

Putcha B-DK, Wright E, Brunzelle JS, Fernandez EJ (2012) Structural basis for negative cooperativity within agonist-bound TR:RXR heterodimers. Proc Natl Acad Sci U S A 109:6084–6087. doi:10.1073/pnas.1119852109

Rastinejad F, Perlmann T, Evans RM, Sigler PB (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature 375:203–211. doi:10.1038/375203a0

Rastinejad F, Wagner T, Zhao Q, Khorasanizadeh S (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. EMBO J 19:1045–1054. doi:10.1093/emboj/19.5.1045

Rochel N, Ciesielski F, Godet J et al (2011) Common architecture of nuclear receptor heterodimers on DNA direct repeat elements with different spacings. Nat Struct Mol Biol 18:564–570. doi:10.1038/nsmb.2054

Sato Y, Ramalanjaona N, Huet T et al (2010) The "Phantom Effect" of the Rexinoid LG100754: Structural and functional insights. PLoS One 5:e15119. doi:10.1371/journal.pone.0015119

Schwabe JW, Chapman L, Finch JT, Rhodes D (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: How receptors discriminate between their response elements. Cell 75:567–578

Shaffer PL, Gewirth DT (2002) Structural basis of VDR-DNA interactions on direct repeat response elements. EMBO J 21:2242–2252. doi:10.1093/emboj/21.9.2242

Shaffer PL, Gewirth DT (2004) Structural analysis of RXR-VDR interactions on DR3 DNA. J Steroid Biochem Mol Biol 89–90:215–219. doi:10.1016/j.jsbmb.2004.03.084

Shaffer PL, McDonnell DP, Gewirth DT (2005) Characterization of transcriptional activation and DNA-binding functions in the hinge region of the vitamin D receptor. Biochemistry (Mosc) 44:2678–2685. doi:10.1021/bi0477182

Suino K, Peng L, Reynolds R et al (2004) The nuclear xenobiotic receptor CAR: Structural determinants of constitutive activation and heterodimerization. Mol Cell 16:893–905. doi:10.1016/j.molcel.2004.11.036

Svensson S, Ostberg T, Jacobsson M et al (2003) Crystal structure of the heterodimeric complex of LXRalpha and RXRbeta ligand-binding domains in a fully agonistic conformation. EMBO J 22:4625–4633. doi:10.1093/emboj/cdg456

Takacs M, Petoukhov MV, Atkinson RA et al (2013) The asymmetric binding of PGC-1a to the ERRα and ERRγ nuclear receptor homodimers involves a similar recognition mechanism. PLoS One 8:e67810. doi:10.1371/journal.pone.0067810

Veesler D, Blangy S, Cambillau C, Sciara G (2008) There is a baby in the bath water: AcrB contamination is a major problem in membrane-protein crystallization. Acta Crystallograph Sect F Struct Biol Cryst Commun 64:880–885. doi:10.1107/S1744309108028248

Velasco LFR, Togashi M, Walfish PG et al (2007) Thyroid hormone response element organization dictates the composition of active receptor. J Biol Chem 282:12458–12466. doi:10.1074/jbc.M610700200

Xu HE, Stanley TB, Montana VG et al (2002) Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARalpha. Nature 415:813–817. doi:10.1038/415813a

Xu RX, Lambert MH, Wisely BB et al (2004) A structural basis for constitutive activity in the human CAR/RXRalpha heterodimer. Mol Cell 16:919–928. doi:10.1016/j.molcel.2004.11.042

Zhang J, Chalmers MJ, Stayrook KR et al (2011) DNA binding alters coactivator interaction surfaces of the intact VDR-RXR complex. Nat Struct Mol Biol 18:556–563. doi:10.1038/nsmb.2046

Zhao Q, Chasse SA, Devarakonda S et al (2000) Structural basis of RXR-DNA interactions. J Mol Biol 296:509–520. doi:10.1006/jmbi.1999.3457

Part II
Nuclear Receptor Co-regulatory Protein Interactions

Primate-Specific Multi-Functional Androgen Receptor Coregulator and Proto-Oncogene Melanoma Antigen-A11 (MAGE-A11)

Elizabeth M. Wilson

1 The Androgen Receptor (AR)

1.1 Structural Constraints on AR Function

The androgen receptor (AR/NR3C4) is a ligand-dependent transcription factor in the steroid receptor family that has distinct DNA binding, ligand binding and transcriptional activation domains (Lubahn et al. 1988a, 1988b; Simental et al. 1991). AR is activated by high affinity binding of testosterone or dihydrotestosterone (DHT) in the hydrophobic core of the highly structured ligand binding domain (LBD) in the carboxyl-terminal region (Sack et al. 2001). DHT is more potent physiologically than testosterone because of its greater hydrophobic character and slower dissociation kinetics (Wilson and French 1976; Askew et al. 2007). AR transcriptional activity depends on binding of the centrally located AR DNA binding domain (DBD) to androgen-response-element DNA sequences in promoter and intron regions of androgen-regulated genes (Lund et al. 1991; Ho et al. 1993; Huang et al. 1999). Structure and function of the AR DBD and LBD depend on strict amino acid sequence conservation and an ordered arrangement of interacting α-helices. The large NH_2-terminal region also important for AR function is largely unstructured, with several short predicted α-helical regions that serve as core interaction sites for coregulators (Simental et al. 1991; He et al. 2004a; Bai et al. 2005; McEwan et al. 2007, Lavery and McEwan 2008). The structural flexibility of the AR NH_2-terminal region facilitates temporal interactions with multiple coregulatory proteins (He et al. 2004a). Interacting partners induce conformational stability

E. M. Wilson (✉)
Laboratories for Reproductive Biology, Departments of Pediatrics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill 27599, USA
e-mail: emw@med.unc.edu

Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, USA

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_8

on intrinsically disordered regions of signaling molecules such as AR that contain short molecular recognition sequences and function in hubs for transcriptional activity (Dunker et al. 2005; Wright and Dyson 2009). An example of multiple binding partners at a shared site is the AR NH_2-terminal F*XX*LF motif that interacts with the highly structured activation function 2 (AF2) in the androgen-bound AR LBD to mediate the AR NH_2- and carboxyl-terminal (N/C) interaction. The same AR F*XX*LF motif serves as the recognition site for melanoma-antigen-A11 (MAGE-A11), a primate-specific AR coregulator (He et al. 2000; Bai et al. 2005).

Initiation of AR transcriptional activity by testosterone or DHT triggers a bipartite nuclear targeting signal located between the DBD and LBD for AR translocation from the cytoplasm to the nucleus (Zhou et al. 1994). Once bound to androgen-response-element DNA, AR transcriptional activity relies on two major androgen-dependent transactivation domains, activation function 1 (AF1) in the intrinsically disordered NH_2-terminal region and AF2 in the highly structured LBD. In the absence of ligand, AR AF1 is inhibited by the unbound LBD through interactions with heat shock proteins released upon androgen binding. The AR AF2 surface of the LBD is stabilized by high affinity androgen binding.

The relative contributions of AF1 and AF2 to AR transcriptional activity depend on locally expressed transcription factors and the androgen-dependent AR N/C interaction. The AF2 hydrophobic surface in the LBD is flanked by oppositely charged amino acid residues and serves as the binding site for the AR NH_2-terminal F*XX*LF motif, F*XX*LF-like motifs in AR coregulators, and L*XX*LL motifs in p160 nuclear receptor coactivators (He et al. 2002b; He and Wilson 2003; Hsu et al. 2003). AR AF2 has a 5–10 fold higher affinity for the AR NH_2-terminal F*XX*LF motif than for p160 coactivator L*XX*LL motifs (He et al. 2004b). Amino acid sequence changes in AF2 during the evolution of human AR within the steroid hormone receptor family favored F*XX*LF over L*XX*LL motif binding, which made the AR NH_2-terminal AF1 the predominant activation domain.

Evolutionary pressure on AR is also evident from AR NH_2-terminal sequence changes within the mammalian lineage that include expansion among primates of the CAG trinucleotide repeat that codes for glutamine (Choong et al. 1998; Choong and Wilson 1998). Human and rodent AR also differ in NH_2-terminal sequence required to interact with MAGE-A11, a primate-specific AR coregulator (Liu et al. 2011). Human and nonhuman primates have a longer AR NH_2-terminal CAG repeat than Old World monkeys. Expansion beyond the normal human AR polymorphic range of 8 to 35 glutamine repeats to 38 to 66 glutamine repeats causes neurotoxicity, interferes with AR expression and results in late onset spinal-bulbar muscular atrophy (La Spada et al. 1991; Choong et al. 1996a; Hong et al. 2006). The disease phenotype is associated with AR aggregation (Merry et al. 1998; Stenoien et al. 1999; Poletti 2004), which could be a consequence of "runaway domain swapping" (Guo and Eisenberg 2006) mediated by the AR N/C interaction. Excessive N/C interactions between monomers may cause degenerative oligomerization of AR (Orr et al. 2010).

Changes in AR NH_2-terminal sequence during primate evolution suggest relatively recent evolutionary pressure on AR that parallels the primate-specific

expression of MAGE-A11. A teleological argument could be made that MAGE-A11 evolved in primates to increase AR transcriptional activity and possibly overcome the detrimental effects of an expanding AR CAG repeat.

1.2 AR NH_2- and Carboxyl-Terminal (N/C) Interaction

The androgen-dependent AR N/C interaction between human AR NH_2-terminal F*XX*LF motif sequence ^{23}FQNLF^{27} and the AF2 surface of the LBD requires high affinity binding of testosterone, DHT or a synthetic anabolic steroid (He et al. 1999, 2000). The androgen-dependent AR N/C interaction is inhibited by classical antagonists such as hydroxyflutamide or casodex (bicalutamide), which are antiandrogens used in the treatment of prostate cancer (Langley et al. 1995; Zhou et al. 1995; Kemppainen and Wilson 1996). Stabilization of AR by high affinity binding of testosterone or DHT is mediated by the N/C interaction and does not occur with partial agonists (Kemppainen et al. 1992, 1999; Langley et al. 1998). Naturally occurring germline mutations in AF2 that disrupt the AR N/C interaction and cause the partial or complete androgen insensitivity syndrome (AIS) (Lim et al. 2000; Quigley et al. 2004) demonstrate the importance of the N/C interaction for AR transcriptional activity *in vivo*.

The AR NH_2-terminal F*XX*LF motif and p160 nuclear receptor coactivator L*XX*LL motifs interact in a competitive relationship at the same AF2 hydrophobic site to regulate AR transcriptional activity (He et al. 2001, 2004). The relative contribution of AR AF1 and AF2 to AR transcriptional activity is therefore modulated by the androgen-dependent AR N/C interaction. Inhibition of p160 coactivator L*XX*LL motif binding to AF2 by the AR F*XX*LF motif shifts AR transcriptional activity from the highly structured AF2 in the LBD which is activated by p160 coactivators to the intrinsically disordered NH_2-terminal AF1 region that interacts with multiple binding partners.

The AR N/C interaction is dynamic and appears to be required for the activation of most but not all androgen-regulated genes (He et al. 2002a; van Royen et al. 2007). The AR N/C interaction mediates an intermolecular interaction between AR monomers that predicts an antiparallel AR dimer (Langley et al. 1995; Schaufele et al. 2005). An intramolecular AR N/C interaction would implicate domain swapping within and between monomers (Bennett et al. 1995). The close correlation between ligands with *in vivo* agonist activity and those that induce the AR N/C interaction provides further support for the N/C domain interaction in AR function. *In vitro* assays in which AR NH_2-terminal and LBD fragments or fusion proteins are coexpressed with androgen-responsive or GAL4 reporter genes provide a measure of agonist or antagonist activity of candidate ligands for potential therapeutic purposes (Langley et al. 1995; Wilson 2011). Partial agonists that do not induce the AR N/C interaction may act as selective AR modulators with beneficial tissue-specific effects in muscle and bone without over-stimulation of the prostate (Schmidt et al. 2009, 2010).

The principal effects of the androgen-dependent N/C interaction are increased AR transcriptional activity through the stabilization of AR by intermolecular interactions between androgen-bound AR monomers and modulation of activity from the major transactivation domains. The intermolecular AR N/C interaction may also provide structural constraints on the AR NH_2-terminal region to stabilize binding regions for coregulator proteins.

1.3 AR Germline and Somatic Mutations

AR is the only nuclear receptor known to be encoded on the human X chromosome. The single AR gene allele in 46XY genetic males causes AR amino acid mutations to have high penetrance. More than 300 naturally occurring AR DBD or LBD germline mutations inhibit AR function and cause AIS (Lubahn et al. 1989; Quigley et al. 1995). The far greater number of missense mutations in the DBD and LBD that disrupt AR function and cause AIS than in the large NH_2-terminal region reflects the structural constraints of DNA and ligand binding and the adaptability of the NH_2-terminal region. The degree to which naturally occurring germline mutations in 46XY genetic males cause partial or complete AIS depends on their position in the sequence, the nature of the altered amino acid, and the extent to which the new amino acid disrupts AR function (Quigley et al. 1995). In some cases, there is not a strict correlation between genotype and phenotype, probably because of individual differences in genetic background that influence AR coregulator expression and circulating androgen levels. Naturally occurring AR germline mutations can be spontaneous in affected newborns or inherited from a 46XX carrier mother who has a normal female phenotype.

A classification scheme was devised to reflect the spectrum of phenotypes in 46XY subjects with partial or complete AIS with different degrees of male genital development (Quigley et al. 1995). Stage 1 AIS is associated with normal male genital development and infertility. Stage 2 AIS is characterized by a hypospadias deformity, where the urethra opens basally rather than at the end of the penis. Hypospadias also occurs in 46XY males without an AR mutation (Hiort et al. 1994). Stage 3 AIS has micropenis or cryptorchidism at birth, where the testes fail to descend from the abdomen. More severe mutations that disrupt AR androgen or DNA binding cause Stage 4 through Stage 7 AIS with increasing loss of male genital development. Stage 7 is the most severe form of AIS that results in a complete external female phenotype in 46XY affected subjects at birth, and absence of pubic and axillary hair later in life (De Bellis et al. 1992; Quigley et al. 1992).

AR missense mutations cause partial or complete AIS with a frequency of about 0.01 % in the population. The phenotypic effects of mutations that disrupt AR function demonstrate the requirement for AR in normal male sex development. AR mutations that cause AIS occur most often in the DBD or LBD, with a lower frequency in the NH_2-terminal region. The low incidence of AR NH_2-terminal missense mutations that cause AIS reflects structural adaptability of the region for

interactions with coregulatory proteins. There are instances, however, where AR NH_2-terminal missense mutations cause AIS. An AR-E2K mutation caused partial AIS by interfering with AR mRNA translation into protein (Choong et al. 1996b). A Stage 3 partial AIS 46XY newborn male had an NH_2-terminal AR-R405S mutation inherited from his mother (Lagarde et al. 2012). The increase in penile growth in response to androgen administration indicated that AR-R405S retained residual activity. The AR-R405S mutation introduced a new serine phosphorylation site that interfered with an increase in AR transcriptional activity associated with MAGE-A11, an AR coregulator that interacts with the AR NH_2-terminal region. Phosphorylation at mutant AR Ser-405 appears to have impaired the effects of MAGE-A11, p300 and/or other protein partners of the AR NH_2-terminal region. Phosphorylation is known to influence the conformational properties of intrinsically disordered regions to either enhance or interfere with interactions of signaling proteins (Wright and Dyson 2009).

In prostate cancer, gain-of-function AR somatic mutations in the ligand binding domain occur with relatively low frequency that may increase in late stage castration-recurrent prostate cancer in association with the genetic instability of cancer (Veldscholte et al. 1990; Harris et al. 1991; Tan et al. 1997; Chang et al. 2001; Gelmann 2002). Some gain-of-function AR somatic mutations expand the range of steroids beyond testosterone and DHT that activate AR. Hydroxyflutamide is an antagonist of wild-type AR, but acts as an agonist with AR-T877A in the LNCaP prostate cancer cell line (Veldscholte et al. 1990; Harris et al. 1991; Chang et al. 2001). AR-H874Y is a somatic mutation identified in the CWR22 human prostate cancer xenograft (Tan et al. 1997). The crystal structure of AR-H874Y showed that the mutant Tyr-874 mediates a direct hydrogen bond that replaced a less stable water-mediated hydrogen bond to wild-type His-874 (He et al. 2006). A consequence of the AR H874Y mutation is stabilization of helix-helix interactions in the LBD that caused testosterone to gain potency and became similar to DHT. Gain-of-function mutations can rescue the effects of some mutations that cause partial AIS (Askew et al. 2012). The predominance of gain rather than loss-of-function AR somatic mutations in prostate cancer supports the importance of AR in prostate cancer growth and progression.

2 AR Coregulator MAGE-A11

2.1 Regulation of Human AR by MAGE-A11

AR transcriptional activity requires the interaction of multiple coactivator proteins with its structured and disordered regions (Heemers and Tindall 2007). MAGE-A11 is a recently identified AR coregulator that interacts with the AR NH_2-terminal region and increases AR transcriptional activity (Bai et al. 2005). MAGE-A11 is a member of the cancer-testis antigen family named originally for its identification

in melanoma (De Plaen et al. 1994; Rogner et al. 1995). MAGE-A11 is expressed at Xq28 in a region of the human X chromosome linked to more than 40 diseases, and there are 32 MAGE family genes coded on the X chromosome (Rogner et al. 1995; Kolb-Kokocinski et al. 2006; Ross et al. 2005). The evolution of MAGE-A11 expression among primates and its function as a human AR coregulator is consistent with more rapidly evolving proteins being involved in reproduction (Swanson and Vacquier 2002), and primate-specific genes preferentially expressed in reproductive organs (Tay et al. 2009). MAGE-A11 was initally identified in human testis as an AR interacting protein (Bai et al. 2005), and is expressed in normal human foreskin fibroblasts, endometrium, benign prostate and prostate cancer (Bai et al. 2005; Karpf et al. 2009). More recent studies have shown that MAGE-A11 is a multi-functional protein required for prostate cancer cell growth (Wilson 2010; Su et al. 2013).

Interaction between AR and MAGE-A11 is mediated by the same AR NH_2-terminal F*XX*LF motif region that mediates the androgen-dependent AR N/C interaction (Bai et al. 2005). This demonstrates a dual function for the AR NH_2-terminal F*XX*LF motif region. AR sequence ^{23}FQNLFQSVREV^{33} required to bind MAGE-A11 is longer than the minimal AR ^{23}FQNLF^{27} sequence that mediates the AR N/C interaction with AR AF2. The longer AR interaction site for MAGE-A11 likely facilitates competition with the androgen-dependent AR N/C interaction to increase AR transcriptional activity. A temporal relationship appears to exist during AR mediated gene activation between the androgen-dependent N/C interaction and AR F*XX*LF motif interaction with MAGE-A11. MAGE-A11 also stabilizes AR in the absence of androgen by interacting with the AR F*XX*LF motif region.

Differences among mammals in the expression of MAGE-A11 and AR NH_2-terminal sequence suggest that the *AR* and *MAGE-A11* genes were subject to convergent evolution. Human and nonhuman primate expression of MAGE-A11 differs from the less evolved mammals such as rats and mice that do not express MAGE-A11. There is also amino acid sequence divergence within the mammalian lineage in AR NH_2-terminal F*XX*LF motif flanking sequence required to interact with MAGE-A11 (Liu et al. 2011). Rat and mouse AR have Ala-33 rather than primate AR Val-33 (Choong et al. 1998). The inability of the human AR-V33A mutant to interact with MAGE-A11 indicates that AR Val-33 in the primate lineage is required to interact with MAGE-A11, and supports the convergent evolution of MAGE-A11 expression with the AR NH_2-terminal interaction site for MAGE-A11 in primates.

MAGE-A11 increases AR transcriptional activity through several mechanisms. MAGE-A11 acts as a molecular bridge between AR dimers. This was shown using AR mutants that lacked the DBD or the AF1 and AF2 transactivation domains, and were inactive when expressed alone in the presence of androgen with or without MAGE-A11 (Minges et al. 2013). However, when these two complementary inactive AR mutants were coexpressed, androgen-dependent transcriptional activity was rescued by MAGE-A11. MAGE-A11 facilitates AR dimer-dimer interactions through an interaction with the AR F*XX*LF motif region in different AR dimers. The model (Fig. 1) is based on evidence that MAGE-A11 itself forms dimers and links AR dimers for increased transcriptional activity through its interaction with the AR

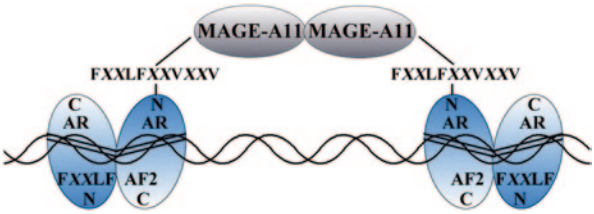


Fig. 1 MAGE-A11 amplification of human AR transcriptional activity by linking AR dimers**.** AR dimerization is mediated by the DNA binding domain and the androgen-dependent AR N/C interaction (Wong et al. 1993). MAGE-A11 binds the AR NH_2-terminal ^{23}F*XX*LF*XX*V*XX*V^{33} motif sequence ^{23}FQNLFQSVREV^{33} (Bai et al. 2005; Liu et al. 2011). The ability of MAGE-A11 to rescue the transcriptional activity of complementary inactive AR mutants that lack DNA binding or transcriptional activation domains suggests that MAGE-A11 dimers link AR dimers (Bai and Wilson 2008, Minges et al. 2013). The ability of MAGE-A11 to increase AR transcriptional activity by linking AR dimers supports the dual function of the AR NH_2-terminal F*XX*LF motif region in the androgen-dependent N/C interaction with activation function 2 in the ligand binding domain and an interaction site for MAGE-A11. This research was originally published in the Journal of Biological Chemistry. Minges et al. 2013 © the American Society for Biochemistry and Molecular Biology

NH_2-terminal F*XX*LF motif (Wong et al. 1993; Bai and Wilson 2008; Minges et al. 2013). The ability of MAGE-A11 to bridge AR dimers supports the dual functions of the AR F*XX*LF motif in mediating the AR N/C interaction and binding MAGE-A11. (Fig. 1)

MAGE-A11 also increases AR transcriptional activity by recruiting p300, a potent acetyltransferase that acetylates histones and transcription factors (Fig. 2) (Askew et al. 2010). MAGE-A11 also helps to recruit p160 nuclear receptor coactivators through a direct interaction and by competing with the AR N/C interaction (Askew et al. 2009). The carboxyl-terminal region of MAGE-A11 contains a MAGE homology domain highly conserved across the MAGE gene family. The MAGE homology domain in MAGE-A11 interacts with the AR F*XX*LF motif region, is modulated by ubiquitination, and contains an F-box-like sequence phosphorylated by the cell cycle checkpoint kinase Chk1 (Bai and Wilson 2008). MAGE-A11 expression also appears to be cell cycle regulated (Bai and Wilson 2008; Askew et al. 2010). The predicted unstructured MAGE-A11 NH_2-terminal region and highly structured carboxyl-terminal region are consistent with the crystal structure of MAGE-G1, another MAGE gene family member. The MAGE-G1 carboxyl-terminal MAGE homology domain contains multiple interacting α-helices linked to an unstructured NH_2-terminal region not detected in the structure analysis (Doyle et al. 2010). (Fig. 2)

2.2 Regulation of Human Progesterone Receptor-B by MAGE-A11

Cancer-testis antigens and the MAGE-A family are sometimes described as expressed exclusively in cancer (Sang et al. 2011). However, proteins expressed in

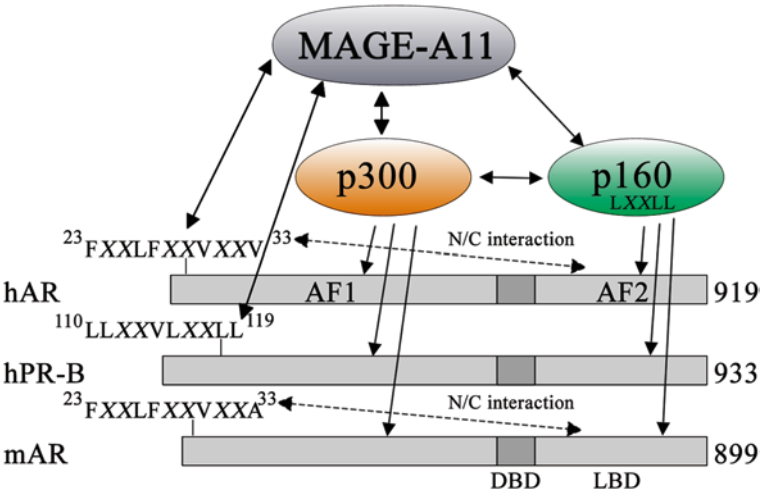


Fig. 2 MAGE-A11 increases transcriptional activation of human AR and human PR-B**.** MAGE-A11 interacts with the human AR (*hAR*) NH_2-terminal F*XX*LF motif region ^{23}F*XX*LF*XX*V*XX*V^{33} and human PR-B (*hPR-B*) NH_2-terminal ^{110}LL*XX*VL*XX*LL^{119} region, and increases transcriptional activity by recruiting p300 and p160 nuclear receptor coactivators. MAGE-A11 binding to the AR F*XX*LF motif region competes with AR F*XX*LF motif binding to activation function 2 (*AF2*) in the ligand binding domain (*LBD*) that mediates the androgen-dependent AR N/C interaction. Mouse AR (*mAR*) and AR from other less evolved mammals have ^{23}F*XX*LF*XX*V*XX*A^{33} NH_2-terminal sequence with Ala-33 instead of Val-33 in primate AR, which undergoes an N/C interaction but does not interact with MAGE-A11. The human AR-V33A mutation inhibits interaction with MAGE-A11. Coevolution of MAGE-A11 and AR is suggested by the primate-specific expression of MAGE-A11 and the primate-specific AR NH_2-terminal sequence required to interact with MAGE-A11. This research was originally published in the Journal of Biological Chemistry. Liu et al. 2011 © the American Society for Biochemistry and Molecular Biology

cancer can have functions in normal cells during development or at times of rapid cell growth or differentiation. The full developmental spectrum of MAGE-A11 expression in humans remains to be examined rigorously. The MAGE-A11 gene promoter contains a CpG island subject to extensive DNA methylation that limits expression in normal tissues. However, there are examples of MAGE-A11 expression in normal tissues of the human reproductive tract.

MAGE-A11 is expressed in normal human endometrium during the menstrual cycle. MAGE-A11 mRNA increases 20-50-fold in mid-secretory endometrial biopsies obtained from normal cycling women (Bai et al. 2008). MAGE-A11 immunostaining increases in mid-secretory endometrial epithelial cells. Highest levels of MAGE-A11 mRNA and protein are in the mid-secretory window of implantation. MAGE-A11 expression in human endometrium increases after the surge in luteinizing hormone (LH) that follows ovulation. Up-regulation of MAGE-A11 mRNA by cyclic AMP in endometrial and prostate cancer cell lines (Bai et al. 2008; Karpf et al. 2009) suggests that the LH-induced increase in cyclic AMP increases MAGE-A11 expression in the mid-secretory endometrium and may be

required to establish receptivity for embryo implantation in humans. The hormone-dependent cycling of human endometrium does not occur in less evolved mammals. The hormone-regulated expression of MAGE-A11 suggests that MAGE-A11 evolved in primates for successful reproduction.

Progesterone receptor (NR3C3)-A (PR-A) and PR-B are isoforms transcribed from the same gene using different promoters (Richer et al. 2002). PR-B is identical to PR-A except for 164 extra NH_2-terminus amino acids. PR-A is the predominant isoform in mouse uterus, whereas the more transcriptionally active PR-B is predominant in human endometrium (Igarashi et al. 2005). MAGE-A11 increases the transcriptional activity of PR-B in an isotype-specific manner by interacting with a unique PR-B NH_2-terminal ^{110}LL*XX*VL*XX*LL^{119} motif (Fig. 2) (Su et al. 2012). The transcriptional enhancing effects of MAGE-A11 for PR-B were not seen with PR-A or the PR-A/B heterodimer. MAGE-A11 interaction with human PR-B is regulated by phosphorylation and ubiquitination, functions synergistically with p300, and amplifies the coregulator effects of p160 nuclear receptor coactivators. Interactions between MAGE-A11 and PR-B may explain the greater transcriptional activity of PR-B relative to PR-A. A ligand-dependent N/C interaction reported for PR-B (Tetel et al. 1999) may be modulated by MAGE-A11.

The coregulator function of MAGE-A11 therefore includes human AR and human PR-B. MAGE-A11 may function primarily as a PR-B coregulator in human endometrium, although effects on AR in uterus have not been excluded. The hormone-regulated cyclic function of human endometrium and evolution of MAGE-A11 is consistent with its function as an amplifying signaling molecule for steroid hormone receptors.

2.3 MAGE-A11 Interacting Partners in the Retinoblastoma Family

Cancer cells interfere with the retinoblastoma (Rb) family of proteins to achieve uncontrolled growth. The Rb family consists of the Rb tumor suppressor, p107 and p130. The major function of the Rb-related proteins is to regulate cell growth by inhibiting the activity of E2F transcription factors that drive the cell cycle. Inactivation of Rb family members in cancer occurs through several mechanisms. Rb gene mutations are common in late stage cancer and block the ability of Rb to inhibit E2F transcription factors resulting in cell cycle progression (Chellappan et al. 1991). DNA tumor viruses, such as adenovirus early protein E1A, inactivate Rb family members to release transcriptionally active E2Fs and promote transition through the G1/S phase of the cell cycle (Fattaey et al. 1993). MAGE-A11 interacts selectively with p107 of the Rb family, stabilizes p107 by inhibition of ubiquitination, and increases the transcriptional activity of E2F1 (Su et al. 2013). MAGE-A11 interacts with hypophosphorylated E2F1 and does not interact with hyperphosphorylated E2F1, suggesting it is directly involved in E2F1 activation.

The ability of MAGE-A11 to increase E2F1 transcriptional activity resembles the activity of adenovirus early protein E1A, a DNA tumor virus protein that promotes cell transformation by disrupting Rb-related protein interactions with E2F transcription factors (Liu and Marmorstein 2007). The specificity of MAGE-A11 interaction with p107, and its ability to increase E2F transcription factor activity, suggest that MAGE-A11 promotes cell cycle progression independent of its function as a steroid hormone receptor coregulator. MAGE-A11 may also provide an important link between steroid receptor transcriptional activity and cell cycle regulation.

3 AR and MAGE-A11 in Prostate Cancer

3.1 AR Activation in Castration-Recurrent Prostate Cancer

Castration-recurrent prostate cancer growth during androgen deprivation therapy is a major clinical challenge. AR continues to drive the growth of castration-recurrent prostate cancer based on the expression of androgen-regulated genes, and inhibition of prostate cancer cell growth by silencing AR (Gregory et al. 1998; Zegarra-Moro et al. 2002; Ponguta et al. 2008). AR coregulators such as p160 nuclear receptor coactivators are often overexpressed in castration-recurrent prostate cancer (Gregory et al. 2001a). However, it remains controversial whether AR splice variants exist and are at sufficiently high levels to impact castration-recurrent prostate cancer (Dehm et al. 2008; Sun et al. 2010). AR splice variants may be largely a phenomenon of prostate cancer cell lines that are subject to extensive gene rearrangements. The level of reported AR variant mRNA in tumor tissue is very low compared to full-length AR (Watson et al. 2010) and could represent incompletely processed RNAs detected using the sensitive technique of quantitative RT-PCR. AR is also highly susceptible to partial proteolysis during isolation, which generates forms that migrate as reported AR variants (Wilson and French 1979; Gregory et al. 2001b).

Continued AR signaling in castration-recurrent prostate cancer could represent to some extent ligand-independent AR activation. However, early studies raised the possibility of intratumoral DHT production from adrenal androgen precursors (Geller et al. 1978). Improved techniques of radioimmunoassay and mass spectrometry demonstrated that testosterone and DHT are at sufficient concentrations to activate AR in castration-recurrent prostate cancer tissue in patients undergoing androgen deprivation therapy (Mohler et al. 2004; Titus et al. 2005; Mostaghel et al. 2007; Montgomery et al. 2008; Locke et al. 2008). Adrenal androgen precursors of DHT synthesis circulate in the blood of patients undergoing androgen deprivation therapy by medical castration. Adrenal androgen supplementation increased prostate DHT in rodent models (Labrie et al. 1988). Normal prostate and prostate cancer cells have enzymes that convert adrenal androgens to DHT (Mohler et al. 2011a, 2011b). Continued reliance on ligand-activated AR in recurrent prostate cancer

growth suggests that adrenal androgens are important precursors for intratumoral DHT synthesis when circulating testosterone and DHT are low in patients undergoing androgen deprivation therapy. It has also been suggested that cholesterol may be a source of intratumoral androgen synthesis in castration-recurrent prostate cancer (Leon et al. 2010; Twiddy et al. 2011; Mostaghel et al. 2012).

3.2 Increased Expression of MAGE-A11 in Castration-Recurrent Prostate Cancer

MAGE-A11 levels increase in prostate cancer during progression to castration-recurrent growth (Karpf et al. 2009). Immunohistochemistry shows an increase in MAGE-A11 protein in the CWR22 human prostate cancer xenograft after castration of tumor-bearing mice and in clinical specimens of castration-recurrent prostate cancer. The increase in MAGE-A11 protein correlates with a 20–100 fold increase in MAGE-A11 mRNA in the CWR22 xenograft with time after castration. MAGE-A11 mRNA can increase up to 1000 fold in clinical specimens of castration-recurrent prostate cancer. An increase in MAGE-A11 mRNA was seen in ~ 30 % of castration-recurrent prostate cancer specimens analyzed, suggesting its overexpression represents one mechanism that contributes to castration-recurrent growth. MAGE-A11 is also overexpressed in epithelial ovarian cancers (James et al. 2013).

The major cause of the increase in MAGE-A11 in prostate and ovarian cancer is hypomethylation of a CpG island at the transcription start site of the X-linked *MAGE-A11* gene promoter (Karpf et al. 2009; James et al. 2013). Progressive hypomethylation of the promoter occurs with time after castration of mice with the CWR22 human prostate cancer xenograft. In a limited number of castration-recurrent prostate cancers analyzed, there was an inverse relationship between AR and MAGE-A11 mRNA levels. One castration-recurrent prostate cancer sample with undetectable AR mRNA determined using quantitative RT-PCR had 1000-fold higher levels of MAGE-A11 mRNA. This suggests a compensatory relationship between AR and MAGE-A11 contributes to prostate cancer growth.

MAGE-A11 mRNA also increases in response to cyclic AMP in human prostate cancer and endometrial cell lines (Bai et al. 2008; Karpf et al. 2009). Cyclic AMP up-regulation of MAGE-A11 is another mechanism that increases MAGE-A11 in castration-recurrent prostate cancer because cyclic AMP levels increase in prostate cancer cells during androgen deprivation (Burchardt et al. 1999). The increase in AR transcriptional activity in response to cyclic AMP (Merkle and Hoffmann 2011) may reflect an increase in MAGE-A11. MAGE-A11 also interacts with and stabilizes the ligand-free AR, which may account in some cases for the increase in AR in castration-recurrent prostate cancer. Higher levels of MAGE-A11 enhance androgen-stimulated AR transcriptional activity through interactions with p300 and p160 coactivators, and drive prostate cancer cell growth through its ability to sequester p107, increase E2F transcriptional activity that drives the cell cycle.

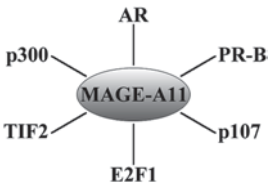


Fig. 3 MAGE-A11 in a transcriptional hub**.** MAGE-A11 interacts with multiple proteins involved in steroid hormone-regulated gene expression and cell cycle control. MAGE-A11 increases human *AR* and human *PR-B* transcriptional activity through direct interactions with NH_2-terminal motifs and through interactions with the p300 acetyltransferase and p160 coactivator, transcriptional intermediary factor 2 (*TIF2*). MAGE-A11 increases cell cycle progression through an interaction with p107 of the retinoblastoma family, which increases *E2F1* transcriptional activity. MAGE-A11 interacts selectively with hypophosphorylated *E2F1*. The multiple interactions of MAGE-A11 suggest its functions in a hub to increase gene transcription and cell cycle progression in primates

3.3 Proto-Oncogene Activity of MAGE-A11 in a Transcriptional Hub

MAGE-A11 is a multifunctional protein expressed at low levels in normal human cells and at higher levels in cancer. Extensive DNA methylation of the promoter that limits MAGE-A11 expression in normal cells is reversed in cancer cells by DNA hypomethylation. The ability of MAGE-A11 to increase the transcriptional activity of AR and PR-B by interacting with unique NH_2-terminal motifs also depends on its interaction with p300 and p160 nuclear receptor coactivators (Fig. 3). MAGE-A11 interacts with p107, a member of the Rb family that regulates cell growth by sequestering E2F transcription factors. MAGE-A11 stabilizes p107 in association with increased E2F1 transcriptional activity. Inhibition of prostate cancer cell growth by suppressing MAGE-A11 expression suggests a critical role in cell cycle regulation. The primate-specific expression of MAGE-A11 suggests it introduces a gain-in-function that enhances steroid hormone receptor transcriptional activity and provides a direct link to cell cycle progression. The multiple interacting partners suggest that MAGE-A11 functions in a transcriptional hub to increase steroid receptor activity and cell cycle progression in rapidly dividing normal cells and in the uncontrolled growth of cancer cells. Based on crystal structure of MAGE-G1 (Doyle et al. 2010), MAGE-A11 may be a hub protein with ordered and disordered regions separated by flexible hinges similar to steroid receptors (Dunker et al. 2005). (Fig. 3)

Acknowlegments John T. Minges provided assistance with the figures and Frank S. French reviewed the manuscript. Research support was from National Institutes of Health Grant HD16910, National Cancer Institute Grant P01-CA77739, and United States Public Health Service grant HD067721 from NIH Eunice Kennedy Shriver NICHD Cooperative Agreement U54-HD35041 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

References

Askew EB, Gampe RT, Stanley TB, Faggart JL, Wilson EM (2007) Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. J Biol Chem 282: 25801–25816

Askew EB, Bai S, Hnat AT, Minges JT, Wilson EM (2009) Melanoma antigen gene protein-A11 (MAGE-11) F-box links the androgen receptor NH_2-terminal transactivation domain to p160 coactivators. J Biol Chem 284:34793–34808

Askew EB, Bai S, Blackwelder AJ, Wilson EM (2010) Transcriptional synergy between melanoma antigen gene protein-A11 (MAGE-11) and p300 in androgen receptor signaling. J Biol Chem 285:21824–21836

Askew EB, Minges JT, Hnat AT, Wilson EM (2012) Structural features discriminate androgen receptor N/C terminal and coactivator interactions. Mol Cell Endocrinol 348:403–410

Bai S, Wilson EM (2008) Epidermal growth factor-dependent phosphorylation and ubiquitinylation of MAGE-11 regulates its interaction with the androgen receptor. Mol Cell Biol 28: 1947–1963

Bai S, He B, Wilson EM (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction. Mol Cell Biol 25:1238–1257

Bai S, Grossman G, Yuan L, Lessey BA, French FS, Young SL, Wilson EM (2008) Hormone control and expression of androgen receptor coregulator MAGE-11 in human endometrium during the window of receptivity to embryo implantation. Mol Hum Reprod 14:107–116

Bennett MJ, Schlunegger MP, Eisenberg D (1995) 3D domain swapping: a mechanism for oligomer assembly. Protein Sci 4:2455–2468

Burchardt T, Burchardt M, Chen MW, Cao Y, de la Taille A, Shabsigh A, Hayek O, Dorai T, Buttyan R (1999) Transdifferentiation of prostate cancer cells to a neuroendocrine cell phenotype in vitro and in vivo. J Urol 162:1800–1805

Chang CY, Walther PJ, McDonnell DP (2001) Glucocorticoids manifest androgenic activity in a cell line derived from a metastatic prostate cancer. Cancer Res 61:8712–8717

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR (1991) The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053–1061

Choong CS, Wilson EM (1998) Trinucleotide repeats in the human androgen receptor: a molecular basis for disease. J Mol Endocrinol 21:235–257

Choong CS, Kemppainen JA, Zhou ZX, Wilson EM (1996a) Reduced androgen receptor gene expression with first exon CAG repeat expansion. Mol Endocrinol 10:1527–1535

Choong CS, Quigley CA, French FS, Wilson EM (1996b) A novel missense mutation in the amino-terminal domain of the human androgen receptor gene in a family with partial androgen insensitivity syndrome causes reduced efficiency of protein translation. J Clin Invest 98:1423–1431

Choong CS, Kemppainen JA, Wilson EM (1998) Evolution of the primate androgen receptor: a structural basis for disease. J Mol Evol 47:334–342

De Bellis A, Quigley CA, Cariello NF, el-Awady MK, Sar M, Lane MV, Wilson EM, French FS (1992) Single base mutations in the human androgen receptor gene causing complete androgen insensitivity: rapid detection by a modified denaturing gradient gel electrophoresis technique. Mol Endocrinol 6:1909–1920

De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora JP, De Smet C et al (1994) Structure, chromosomal localization, and expression of 12 genes of the MAGE family. Immunogenetics 40:360–369

Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ (2008) Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. Cancer Res 68:5469–5477

Doyle JM, Gao J, Wang J, Yang M, Potts PR (2010) MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell 39:963–974

Dunker AK, Cortese MS, Romero P, Iakoucheva LM, Uversky VN (2005) Flexible nets. The roles of intrinsic disorder in protein interaction networks. FEBS J 272:5129–5148

Fattaey AR, Harlow E, Helin K (1993) Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. Mol Cell Biol 13:7267–7277
Geller J, Albert J, Loza D, Geller S, Stoeltzing W, de la Vega D (1978) DHT concentrations in human prostate cancer tissue. J Clin Endocrinol Metab 46:440–444
Gelmann EP (2002) Molecular biology of the androgen receptor. J Clin Oncol 20:3001–3015
Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL, French FS (1998) Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. Cancer Res 58:5718–5724
Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS, Wilson EM (2001a) A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 61:4315–4319
Gregory CW, He B, Wilson EM (2001b) The putative androgen receptor-A form results from in vitro proteolysis. J Mol Endocrinol 27:309–319
Guo Z, Eisenberg D (2006) Runaway domain swapping in amyloid-like fibrils of T7 endonuclease I. Proc Natl Acad Sci U S A 103:8042–8047
Harris SE, Harris MA, Rong Z, Hall J, Judge S, French FS, Joseph DR, Lubahn DB, Simental JA, Wilson EM (1991) Androgen regulation of HBGF-1 (alpha FGF) mRNA and characterization of the androgen receptor mRNA in the human prostate carcinoma cell line LNCaP/A-Dep. In: Karr JP, Coffey DS, Smith RG, Tindall DJ (eds) Molecular and cellular biology of prostate cancer. Plenum, New York, pp 315–330
He B, Wilson EM (2003) Electrostatic modulation of steroid receptor recruitment of the LXXLL and FXXLF motifs. Mol Cell Biol 23:2135–2150
He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM (1999) Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH_2-terminal domain. J Biol Chem 274:37219–37225
He B, Kemppainen JA, Wilson EM (2000) FXXLF and WXXLF sequences mediate the NH_2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986–22994
He B, Bowen NT, Minges JT, Wilson EM (2001) Androgen-induced NH_2- and carboxyl-terminal interaction inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 276:42293–42301
He B, Lee LW, Minges JT, Wilson EM (2002a) Dependence of selective gene activation on the androgen receptor NH_2- and carboxyl-terminal interaction. J Biol Chem 277:25631–25639
He B, Minges JT, Lee LW, Wilson EM (2002b) The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226–10235
He B, Bai S, Hnat AT, Kalman RI, Minges JT, Patterson C, Wilson EM (2004a) An androgen receptor NH_2-terminal conserved motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP). J Biol Chem 279:30643–30653
He B, Gampe RT, Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM (2004b) Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425–438
He B, Gampe RT, Hnat AT, Faggart JL, Minges JT, French FS, Wilson EM (2006) Probing the functional link between androgen receptor coactivator and ligand binding sites in prostate cancer and androgen insensitivity. J Biol Chem 281:6648–6663
Heemers HV, Tindall DJ (2007) Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 28:778–808
Hiort O, Klauber G, Cendron M, Sinnecker GH, Keim L, Schwinger E, Wolfe HJ, Yandell DW (1994) Molecular characterization of the androgen receptor gene in boys with hypospadias. Eur J Pediatr 153:317–321
Ho KC, Marschke KB, Tan J, Power SG, Wilson EM, French FS (1993) A complex response element in intron 1 of the androgen-regulated 20-kDa protein gene displays cell type-dependent androgen receptor specificity. J Biol Chem 268:27226–27235

Hong KW, Hibino E, Takenaka O, Hayasaka I, Murayama Y, Ito S, Inoue-Murayama M (2006) Comparison of androgen receptor CAG and GGN repeat length polymorphism in humans and apes. Primates 47:248–254

Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C (2003) The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W) XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691–23698

Huang W, Shostak Y, Tarr P, Sawyers C, Carey M (1999) Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. J Biol Chem 274:25756–25768

Igarashi TM, Bruner-Tran KL, Yeaman GR, Lessey BA, Edwards DP, Eisenberg E, Osteen KG (2005) Reduced expression of progesterone receptor-B in the endometrium of women with endometriosis and in cocultures of endometrial cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fertil Steril 84:67–74

James SR, Cedeno CD, Sharma A, Zhang W, Mohler JL, Odunsi K, Wilson EM, Karpf AR (2013) DNA methylation and nucleosome occupancy regulate the cancer germline antigen gene MAGEA11. Epigenetics 8:849–863

Karpf AR, Bai S, James SR, Mohler JL, Wilson EM (2009) Increased expression of androgen receptor coregulator MAGE-11 in prostate cancer by DNA hypomethylation and cyclic AMP. Mol Cancer Res 7:523–535

Kemppainen JA, Wilson EM (1996) Agonist and antagonist activities of hydroxyflutamide and casodex relate to androgen receptor stabilization. Urology 48:157–163

Kemppainen JA, Lane MV, Sar M, Wilson EM (1992) Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation: specificity for steroids and antihormones. J Biol Chem 267:968–974

Kemppainen JA, Langley E, Wong CI, Bobseine K, Kelce WR, Wilson EM (1999) Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. Mol Endocrinol 13:440–454

Kolb-Kokocinski A, Mehrle A, Bechtel S, Simpson JC, Kioschis P, Wiemann S, Wellenreuther R, Poustka A (2006) The systematic functional characterisation of Xq28 genes prioritises candidate disease genes. BMC Genomics 7:29

La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77–79

Labrie C, Belanger A, Labrie F (1988) Androgenic activity of dehydroepiandrosterone and androstenedione in the rat ventral prostate. Endocrinology 123:1412–1417

Lagarde WH, Blackwelder AJ, Minges JT, Hnat AT, French FS, Wilson EM (2012) Androgen receptor exon 1 mutation causes androgen insensitivity by creating a phosphorylation site and inhibiting MAGE-A11 activation of NH_2- and carboxyl-terminal interaction-dependent transactivation. J Biol Chem 287:10905–10915

Langley E, Zhou ZX, Wilson EM (1995) Evidence for an antiparallel orientation of the ligand activated human androgen receptor dimer. J Biol Chem 270:29983–29990

Langley E, Kemppainen JA, Wilson EM (1998) Intermolecular NH_2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. J Biol Chem 273:92–101

Lavery DN, McEwan IJ (2008) Structural characterization of the native NH_2-terminal transactivation domain of the human androgen receptor: a collapsed disordered conformation underlies structural plasticity and protein-induced folding. Biochemistry 47:3360–3369

Leon CG, Locke JA, Adomat HH, Etinger SL, Twiddy AL, Neumann RD, Nelson CC, Guns ES, Wasan KM (2010) Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model. Prostate 70:390–400

Lim J, Ghadessy FJ, Abdullah AA, Pinsky L, Trifiro M, Yong EL (2000) Human androgen receptor mutation disrupts ternary interactions between ligand, receptor domains, and the coactivator TIF2 (transcription intermediary factor 2). Mol Endocrinol 14:1187–1197

Liu X, Marmorstein R (2007) Structure of the retinoblastoma protein bound to adenovirus E1A reveals the molecular basis for viral oncoprotein inactivation of a tumor suppressor. Genes Dev 21:2711–2716

Liu Q, Su S, Blackwelder AJ, Minges JT, Wilson EM (2011) Gain in transcriptional activity by primate-specific coevolution of melanoma antigen-A11 and its interaction site in androgen receptor. J Biol Chem 286:29951–29963

Locke JA, Guns ES, Lubik AA, Adomat HH, Hendy SC, Wood CA, Ettinger SL, Gleave ME, Nelson CC (2008) Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 68:6407–6415

Lubahn DB, Joseph DR, Sar M, Tan JA, Higgs HN, Larson RE, French FS, Wilson EM (1988a) The human androgen receptor: complementary DNA cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 2:1265–1275

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM (1988b) Cloning of human androgen receptor cDNA and localization to the X chromosome. Science 240: 327–330

Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS (1989) Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc Natl Acad Sci U S A 86:9534–9538

Lund SD, Gallagher PM, Wang B, Porter SC, Ganschow RE (1991) Androgen responsiveness of the murine beta-glucuronidase gene is associated with nuclease hypersensitivity, protein binding, and haplotype-specific sequence diversity within intron 9. Mol Cell Biol 11:5426–5434

McEwan IJ, Lavery D, Fischer K, Watt K (2007) Natural disordered sequences in the amino terminal domain of nuclear receptors: lessons from the androgen and glucocorticoid receptors. Nucl Recept Signal 5:e001

Merkle D, Hoffmann R (2011) Roles of cAMP and cAMP-dependent protein kinase in the progression of prostate cancer: cross-talk with the androgen receptor. Cell Signal 23:507–515

Merry DE, Kobayashi Y, Bailey CK, Taye AA, Fischbeck KH (1998) Cleavage, aggregation and toxicity of the expanded androgen receptor in spinal and bulbar muscular atrophy. Hum Mol Genet 7:693–701

Minges JT, Su S, Grossman G, Blackwelder AJ, Pop EA, Mohler JL, Wilson EM (2013) Melanoma antigen-A11 (MAGE-A11) enhances transcriptional activity by linking androgen receptor dimers. J Biol Chem 288:1939–1952

Mohler JL, Titus MA, Bai S, Kennerley BJ, Lih FB, Tomer KB, Wilson EM (2011a) Activation of the androgen receptor by intratumoral bioconversion of androstanediol to dihydrotestosterone in prostate cancer. Cancer Res 71:1486–1496

Mohler JL, Titus MA, Wilson EM (2011b) Potential prostate cancer drug target: bioactivation of androstanediol by conversion to dihydrotestosterone. Clin Cancer Res 17:5844–5849

Mohler JL, Gregory CW, Ford OH, Kim D, Weaver CM, Petrusz P, Wilson EM, French FS (2004) The androgen axis in recurrent prostate cancer. Clin Cancer Res 10:440–448

Montgomery RB, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, True LD, Nelson PS (2008) Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res 68:4447–4454

Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS (2007) Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res 67:5033–5041

Mostaghel EA, Solomon KR, Pelton K, Freeman MR, Montgomery RB (2012) Impact of circulating cholesterol levels on growth and intratumoral androgen concentration of prostate tumors. PLoS One 7:e30062

Orr CR, Montie HL, Liu Y, Bolzoni E, Jenkins SC, Wilson EM, Joseph JD, McDonnell DP, Merry DE (2010) An interdomain interaction of the androgen receptor is required for its aggregation and toxicity in spinal and bulbar muscular atrophy. J Biol Chem 285:35567–35577

Poletti A (2004) The polyglutamine tract of androgen receptor: from functions to dysfunctions in motor neurons. Front Neuroendocrinol 25:1–26

Ponguta LA, Gregory CW, French FS, Wilson EM (2008) Site-specific androgen receptor serine phosphorylation linked to epidermal growth factor-dependent growth of castration-recurrent prostate cancer. J Biol Chem 283:20989–21001

Quigley CA, Evans BA, Simental JA, Marschke KB, Sar M, Lubahn DB, Davies P, Hughes IA, Wilson EM, French FS (1992) Complete androgen insensitivity due to deletion of exon C of the androgen receptor gene highlights the functional importance of the second zinc finger of the androgen receptor in vivo. Mol Endocrinol 6:1103–1112

Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS (1995) Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16:271–321

Quigley CA, Tan JA, He B, Zhou ZX, Mebarki F, Morel Y, Forest MG, Chatelain P, Ritzén EM, French FS, Wilson EM (2004) Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH_2- and carboxyl-terminal interaction. Mech Ageing Dev 125:683–695

Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB (2002) Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem 277:5209–5218

Rogner UC, Wilke K, Steck E, Korn B, Poustka A (1995) The melanoma antigen gene (MAGE) family is clustered in the chromosomal band Xq28. Genomics 29:725–731

Ross MT, Grafham DV, Coffey AJ, Scherer S, McLay K, Muzny D et al (2005) The DNA sequence of the human X chromosome. Nature 434:325–337

Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR, Weinmann R, Einspahr HM (2001) Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci U S A 98:4904–4909

Sang M, Lian Y, Zhou X, Shan B (2011) MAGE-A family: attractive targets for cancer immunotherapy. Vaccine 29:8496–8500

Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, Miner JN, Diamond MI (2005) The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. Proc Natl Acad Sci U S A 102:9802–9807

Schmidt A, Harada S, Kimmel DB, Bai C, Chen F, Rutledge SJ, Vogel RL, Scafonas A, Gentile MA, Nantermet PV, McElwee-Witmer S, Pennypacker B, Masarachia P, Sahoo SP, Kim Y, Meissner RS, Hartman GD, Duggan ME, Rodan GA, Towler DA, Ray WJ (2009) Identification of anabolic selective androgen receptor modulators with reduced activities in reproductive tissues and sebaceous glands. J Biol Chem 284:36367–36376

Schmidt A, Kimmel DB, Bai C, Scafonas A, Rutledge S, Vogel RL, McElwee-Witmer S, Chen F, Nantermet PV, Kasparcova V, Leu CT, Zhang HZ, Duggan ME, Gentile MA, Hodor P, Pennypacker B, Masarachia P, Opas EE, Adamski SA, Cusick TE, Wang J, Mitchell HJ, Kim Y, Prueksaritanont T, Perkins JJ, Meissner RS, Hartman GD, Freedman LP, Harada S, Ray WJ (2010) Discovery of the selective androgen receptor modulator MK-0773 using a rational development strategy based on differential transcriptional requirements for androgenic anabolism versus reproductive physiology. J Biol Chem 285:17054–17064

Simental JA, Sar M, Lane MV, French FS, Wilson EM (1991) Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 266:510–518

Stenoien DL, Cummings CJ, Adams HP, Mancini MG, Patel K, DeMartino GN, Marcelli M, Weigel NL, Mancini MA (1999) Polyglutamine-expanded androgen receptors form aggregates that sequester heat shock proteins, proteasome components and SRC-1, and are suppressed by the HDJ-2 chaperone. Hum Mol Genet 8:731–741

Su S, Blackwelder AJ, Grossman G, Minges JT, Yuan L, Young SL, Wilson EM (2012) Primate-specific melanoma antigen-A11 regulates isoform-specific human progesterone receptor-B transactivation. J Biol Chem 287:34809–34824

Su S, Minges JT, Grossman G, Blackwelder AJ, Mohler JL, Wilson EM (2013) Proto-oncogene activity of melanoma antigen-A11 (MAGE-A11) regulates retinoblastoma-related p107 and E2F1 proteins. J Biol Chem 288:24809–24824

Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, Page ST, Coleman IM, Nguyen HM, Sun H, Nelson PS, Plymate SR (2010) Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest 120:2715–2730

Swanson WJ, Vacquier VD (2002) The rapid evolution of reproductive proteins. Nat Rev Genet 3:137–144

Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL, French FS (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. Mol Endocrinol 11:450–459

Tay SK, Blythe J, Lipovich L (2009) Global discovery of primate-specific genes in the human genome. Proc Natl Acad Sci U S A 106:12019–12024

Tetel MJ, Giangrande PH, Leonhardt SA, McDonnell DP, Edwards DP (1999) Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. Mol Endocrinol 13:910–924

Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL (2005) Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. Clin Cancer Res 11:4653–4657

Twiddy AL, Leon CG, Wasan KM (2011) Cholesterol as a potential target for castration-resistant prostate cancer. Pharm Res 28:423–437

van Royen ME, Cunha SM, Brink MC, Mattern KA, Nigg AL, Dubbink HJ, Verschure PJ, Trapman J, Houtsmuller AB (2007) Compartmentalization of androgen receptor protein-protein interactions in living cells. J Cell Biol 177:63–72

Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 173:534–540

Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, Kim K, Sawyers CL (2010) Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc Natl Acad Sci U S A 107:16759–16765

Wilson EM (2010) Androgen receptor molecular biology and potential targets in prostate cancer. Therap Adv Urol 2:105–117

Wilson EM (2011) Analysis of interdomain interactions of the androgen receptor. Methods Mol Biol 776:113–129

Wilson EM, French FS (1976) Binding properties of androgen receptors: evidence for identical receptors in rat testis, epididymis, and prostate. J Biol Chem 251:5620–5629

Wilson EM, French FS (1979) Effects of proteases and protease inhibitors on the 4.5S and 8S androgen receptor. J Biol Chem 254:6310–6319

Wong CI, Zhou ZX, Sar M, Wilson EM (1993) Steroid requirement for androgen receptor dimerization and DNA binding: modulation by intramolecular interactions between the NH_2-terminal and steroid binding domains. J Biol Chem 268:19004–19012

Wright PE, Dyson HJ (2009) Linking folding and binding. Curr Opin Struct Biol 19:31–38

Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ (2002) Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 62: 1008–1013

Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM (1994) A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor: requirement for the DNA binding domain and modulation by the NH_2-terminal and carboxyl-terminal sequences. J Biol Chem 269: 13115–13123

Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM (1995) Specificity of ligand dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 9:208–218

Assembly and Regulation of Nuclear Receptor Corepressor Complexes

Christopher J. Millard and John W. R. Schwabe

1 Introduction

Gene expression in eukaryotes is a tightly controlled process that involves the recruitment of many large coregulator complexes to chromatin so as to regulate transcription. Coregulator complexes have historically been classed as either coactivator or corepressor complexes, but this classification has been muddied with time due to the emerging complexity of the roles of coregulator complexes (reviewed in (McKenna et al. 1999) and (Lonard and O'Malley 2007)).

Indeed, whilst many corepressor complexes function to repress transcription, the role of corepressors can be reversed on negatively regulated genes (Tagami et al. 1999; Santos et al. 2011). Furthermore, emerging evidence suggests that corepressor complexes may also be recruited to actively transcribed genes so as to prevent inappropriate initiation of transcription within the body of the gene or to prime genes for further rounds of transcription (Métivier et al. 2003; Wang et al. 2009).

Many coregulator complexes, as their name implies, are recruited to the genome through association with specific transcription factors or families of transcription factors. However, many of these complexes contain intrinsic DNA and chromatin binding activities and therefore they may also play a role in regulating chromatin structure independently of specific transcription factors. Furthermore there is increasing evidence that these complexes are involved in other processes involving chromatin such as DNA replication and repair (Doyon et al. 2006; Qin and Parthun 2006; Kouzarides 2007).

Coregulator complexes appear to function through the recruitment of chromatin modifying or remodelling activities to the genome. These activities include ATP-dependent nucleosome re-positioning as well as enzymes that add or remove covalent "epigenetic" modifications to both DNA and histones. These modifications

J. W. R. Schwabe (✉) · C. J. Millard
Henry Wellcome Laboratories of Structural Biology, Department of Biochemistry, University of Leicester, Leicester LE1 9HN, UK
e-mail: john.schwabe@le.ac.uk

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_9

include DNA-methylation, histone methylation and acetylation along with many others. These activities are often referred to as epigenetic "writers" and "erasers" (Ruthenburg et al. 2007). Coregulator complexes also contain domains that can "read" these epigenetic modifications (reviewed in (Musselman et al. 2012)).

This review will focus largely on the two nuclear corepressor proteins NCoR and SMRT that associate with histone deacetylase enzymes (HDACs) to control gene expression. The removal of acetyl groups from histone tails by HDACs is associated with gene repression (Turner 1993; Taunton et al. 1996; Finnin et al. 1999). In addition to SMRT and NCoR there are a number of other corepressor complexes that have been implicated in regulation of transcription by nuclear receptors including: LCoR, RIP140, and HDAC containing complexes such as SIN3a, CoREST and NuRD (Cavaillès et al. 1995; Mathur et al. 2001; Kumar et al. 2002; Fernandes et al. 2003; Metzger et al. 2005). However their roles in relation to nuclear receptor signalling is less-well understood.

2 The Anatomy of Nuclear Receptor Corepressors

NCoR and SMRT (aka NCOR1 and NCOR2) are the best-characterised corepressors and are important for transcriptional repression by nuclear receptors. These homologous platform proteins are 40% identical and were originally identified through their interaction with unliganded retinoid and thyroid hormone receptors (Chen and Evans 1995; Hörlein et al. 1995). NCoR and SMRT share many similar functions, but importantly are not completely redundant, since whole-body knockout of either gene is embryonically lethal (Jepsen et al. 2000; Jepsen et al. 2007). Genetic deletion of NCoR results in defects in CNS, erythrocyte and thymocyte development whereas deletion of SMRT causes brain and heart defects. These differences in phenotype may be due to distinct cell-type specific expression patterns. For example in thymocytes, NCoR expression is detectable, whereas SMRT is not expressed. In cells from the forebrain, SMRT mRNA expression levels are considerably higher than those of NCoR mRNA (Jepsen et al. 2000; Jepsen et al. 2007). The phenotypic differences may also be due to differential recruitment of NCoR and SMRT to target genes by nuclear receptors. RAR has been shown in numerous biochemical studies to preferentially recruit SMRT, whereas TR preferentially recruits NCoR (Hu and Lazar 1999; Webb et al. 2000; Cohen et al. 2001; Makowski et al. 2003). Despite the different roles in development, at the molecular level the two corepressors assemble into very similar complexes with common interaction partners (Fig. 1).

SMRT and NCoR are large proteins (~2500 residues) that interact with many nuclear receptors, other transcription factors, histone deacetylases and other scaffold proteins. The amino-terminus of SMRT (residues 168–725) is the most structured region of the protein; is highly conserved between SMRT and NCoR (68% identity) and forms the core of the repression complex. In contrast, the carboxy-terminal region of the protein (c. 1700 residues) contains almost no predicted secondary

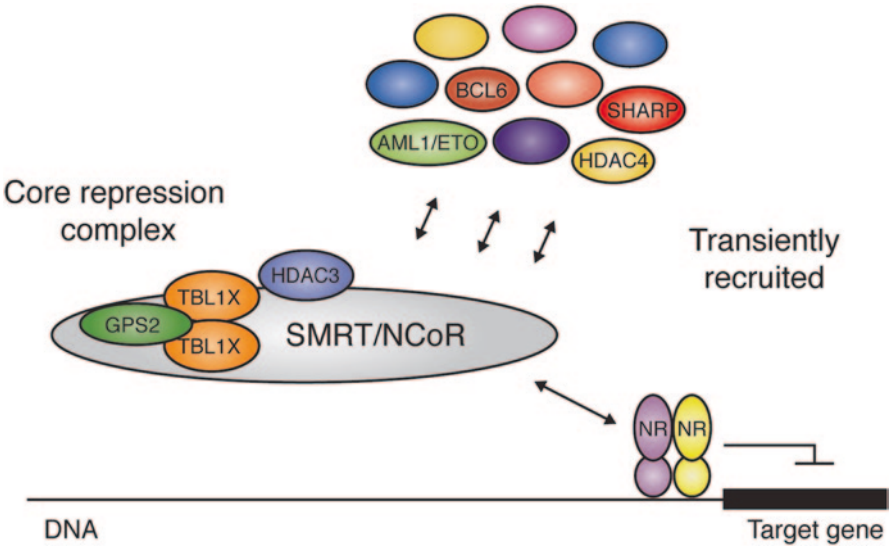


Fig. 1 The core SMRT/NCoR complex is recruited to chromatin through the ligand-binding domains of unliganded-nuclear receptors to repress transcription. The SMRT/NCoR complex transiently assembles with chromatin modifying enzymes and other factors to form a large protein complex that regulates gene expression

structure and in large part is predicted to be intrinsically disordered (i.e. lacks an intrinsically fixed structure) (Fig. 2).

Within the amino-terminal region, there are two structured SANT domains. The amino-terminal domain has been shown to be essential for recruitment of histone deacetylase 3 (Wen et al. 2000; Guenther et al. 2001). In contrast the second SANT domain has been reported to mediate interactions with histones (Yu et al. 2003; Hartman et al. 2005). Given that the two SANT domains are 36% identical and 69% similar, it seems likely that they arose by domain duplication followed by functional divergence (Boyer et al. 2004). Interestingly, both domains have a basic charged surface suggesting that if the second SANT domain is mediating interactions with histones, it may also be interacting with negatively charged DNA wrapped around the histone octamer.

The region of SMRT and NCoR amino-terminal to the first SANT domain contains the region that has been shown to be responsible for the recruitment of the proteins GPS2 and TBL1X which form the core scaffold of the repression complex (Guenther et al. 2000; Zhang et al. 2002).

Throughout the apparently unstructured carboxy-terminal region of the corepressors there are short stretches of residues that are conserved between SMRT and NCoR. Several of these have been shown to act as interaction motifs for transcription factors and other proteins including unliganded or antagonist-bound nuclear receptors. These sequence motifs seem to become structured upon forming a complex with their respective partner proteins (see below).

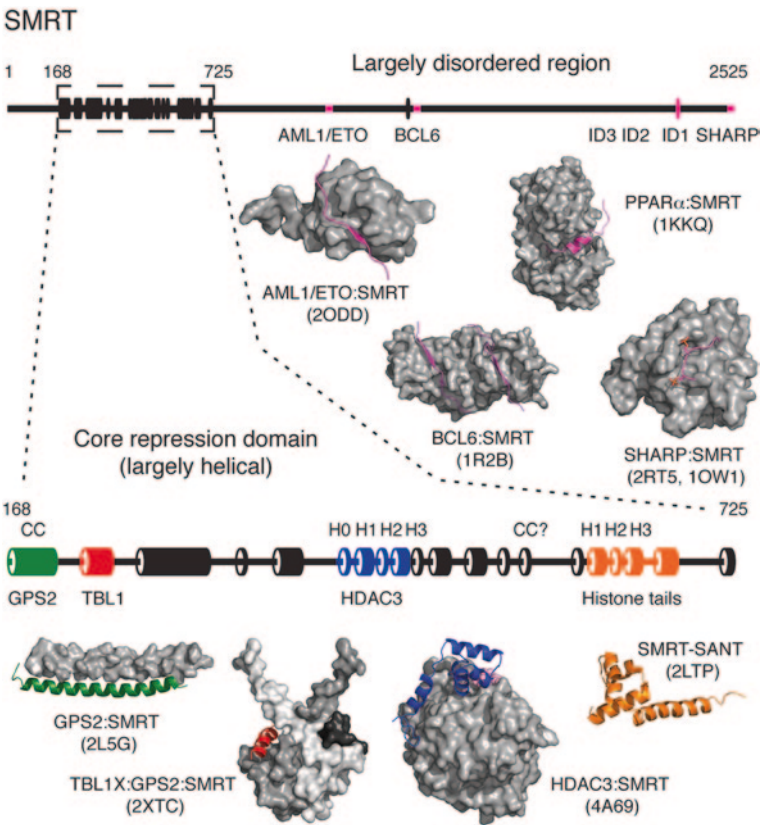


Fig. 2 The nuclear receptor corepressor SMRT is largely intrinsically disordered except for the core repression domain found towards the amino-terminus of the protein (residues 168–725). The secondary structure prediction of SMRT is shown with α-helices depicted as cylinders and with the core repression domain enlarged for clarity (prediction made using http://bioinf.cs.ucl.ac.uk/psipred). Structural characterisation of SMRT has been most successful through the study of SMRT fragments in complex with other proteins from the SMRT corepressor complex. Structures are illustrated with SMRT coloured to match the secondary structure prediction and shown as cartoon

Although SMRT and NCoR are the best characterised of the nuclear receptor corepressors, a great many other proteins have been implicated in mediating transcriptional repression by nuclear receptors. Some of these, such as RIP140, Hairless and LCoR, act as repressors of agonist bound nuclear receptors and probably serve to attenuate activation (Cavaillès et al. 1995; Potter et al. 2001; Fernandes et al. 2003). Like SMRT and NCoR, these proteins are in large part intrinsically disordered suggesting that this is a functionally important characteristic of this family of proteins.

3 The Core SMRT/NCoR Repression Complex

When SMRT or NCoR are purified from cells, three proteins (GPS2, TBL1X and HDAC3) invariably co-purify as a complex with the corepressor (Guenther et al. 2000; Li et al. 2000). This complex shows resistance to dissociation and is stable in the presence of high salt, moderate sodium dodecyl sulfate and non-ionic or ionic detergents suggesting that it acts as a stable core to the repression complex (Zhang et al. 2002). HDAC3 is a histone deacetylase with a well-established role in nuclear receptor mediated transcriptional repression (Yang et al. 1997; Dangond et al. 1998). GPS2 is a G-protein signalling regulator, initially discovered in a yeast pheromone response pathway, and shown to be involved in MAP kinase cascades (Spain et al. 1996). GPS2 has been shown to have an important role in hepatic bile acid synthesis and promotes adipose tissue inflammation in obese subjects (Sanyal et al. 2007; Toubal et al. 2013). Mutations in GPS2 have also been linked to medulloblastoma (Pugh et al. 2012).

TBL1X (and its closely related homologues TBL1Y and TBL1XR1) are WD40 repeat-containing proteins that have been shown to be involved in human hearing; loss-of-function mutations in TBL1X have been linked to deafness (Bassi et al. 1999). TBL1X has been shown be important for maintaining a healthy liver fat content through interaction with PPARα; TBL1X deficiency results in fatty liver development and further metabolic syndromes such as steatosis and hypertriglyceridemia (Kulozik et al. 2011).

HDAC3, GPS2 and TBL1X interact with the highly conserved core-region of SMRT (168–725). Residues 168–297 are sufficient to bind to both GPS2 and TBL1X, whereas residues 409–475 are required for the recruitment of HDAC3 (Oberoi et al. 2011). In between these two interaction domains there are three predicted α-helices that may also contribute to the interaction with the TBL1X and/or HDAC3. Importantly it has been shown that there is a three-way complex between SMRT, GPS2 and TBL1X, such that SMRT and GPS2 interact directly with each other as well as both interacting with TBL1X, thus forming a tight three-way complex (Oberoi et al. 2011).

Structural studies have demonstrated that residues 167–207 of SMRT form an anti-parallel coiled-coil with residues 53–90 of GPS2 (Fig. 3) (Oberoi et al. 2011). The anti-parallel orientation positions the two regions that bind to TBL1X at one end of the coiled coil. Residues 227–297 of SMRT and 1–52 of GPS2 interact with the amino-terminal domain of TBL1X. Modelling, together with interaction mapping studies, has shown that the interaction regions in SMRT and GPS2 form short helical structures that bind in grooves on either side of a TBL1X dimer.

TBL1X consists of a LisH domain and a WD40 domain. The LisH domain of TBL1X forms a homodimer with an antiparallel four-helix bundle stabilised by polar and non-polar contacts. Two additional helices cross over to form an X-shaped structure that rests on the four-helix bundle. Two TBL1X dimers interact through one surface of the four-helix bundle so as to form a tetramer (Oberoi et al. 2011).

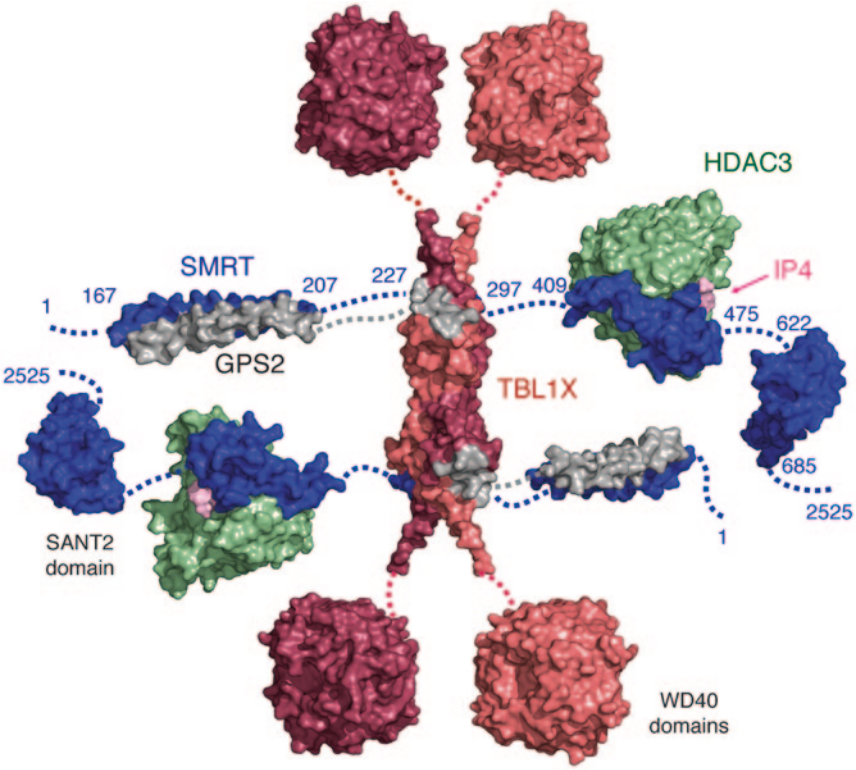


Fig. 3 A schematic model of the core repression domain of SMRT (*blue*) showing the characterised interactions with HDAC3 (*green*), IP4 (*pink*), GPS2 (*grey*) and TBL1X (*salmon* and *red*). SMRT is arranged in a linear format for simplicity but may fold to a more compact arrangement in solution. SMRT:GPS2 (pdbcode 2LG5), TBL1X tetramer (pdbcode 2XTC), WD40 domains of TBLXR1(pdbcode 4LG9), HDAC3:SMRT-SANT1 (pdbcode 4A69, 1XC5), SMRT-SANT2 (pdbcode 2LTP). *Dotted lines* indicate regions of SMRT that have not been structurally characterised

The similarity between TBL1X, TBL1Y and TBL1XR1 is such that it would be expected that a TBL1 tetramer could be formed from any combination of these closely related proteins.

An eight-bladed WD40 domain is located carboxy-terminal to the TBL1X tetramerisation domain. Thus the tetramerisation of TBL1X amino-terminal domain draws together four WD40 domains into close proximity (which may have implications for chromatin targeting). The WD40 domain structure of TBLR1X has been solved (PDBcode 4LG9) and resembles the WD40 domain from WDR5 that is known to mediate interactions with chromatin (Wysocka et al. 2005; Couture et al. 2006; Ruthenburg et al. 2006). It is possible that the WD40 domains in TBL1X will serve a similar role.

The tetramerisation of TBL1X also implies that the whole complex will contain 2 copies of SMRT or NCoR, 2 copies of HDAC3 and 2 of GPS2 along with the

TBL1X tetramer. This would equate to a total molecular weight of approximate 1 MDa that fits well with the reported size of the complex when purified from nuclear extracts (Guenther et al. 2000; Li et al. 2000; Varlakhanova et al. 2011).

4 Assembly of HDAC3 in the SMRT/NCoR Complex

As mentioned earlier, the first of the two SANT domains in SMRT and NCoR has been shown to be responsible for the recruitment of HDAC3. Surprisingly, it was also found that enzymatic activity of HDAC3 was very significantly enhanced through interaction with this domain which was named accordingly: "the deacetylase activation domain" (DAD) (Guenther et al. 2001). The SMRT-DAD is in fact an extended SANT domain which was shown by NMR to fold into a compact four-helical structure composed of a canonical three-helix bundle SANT domain, and amino-terminal helix termed H0 (Codina et al. 2005). Structure-guided mutagenesis was used to determine the residues that were required for binding and those that were essential for activating HDAC3. The molecular detail of this interaction was clarified through the crystal structure of the SMRT-DAD bound to HDAC3 (Watson et al. 2012). The structure shows that the isolated SMRT-DAD must undergo a major structural rearrangement on binding. Helix H0 unfolds to expose an HDAC3 binding surface, and both helix H0 and the SANT domain make extensive intermolecular interactions with the surface of HDAC3. Whether this unfolding transition occurs on binding HDAC3 or whether the complex is assembled directly after synthesis is uncertain.

5 HDAC Activity Is Regulated by Inositol Phosphates

The structure of the HDAC3:SMRT complex led to the surprising finding that there was an inositol tetrakisphosphate molecule (Ins(1,4,5,6)P_4) located at the interface of HDAC and the SANT domain of the corepressor (Watson et al. 2012). The inositol phosphate co-purified with the complex that had been expressed in HEK293 cells. Subsequent deacetylase assays revealed that the IP_4 can be washed out of the HDAC3:SMRT complex using high salt resulting in an enzymatically inert complex (Millard et al. 2013). Addition of inositol phosphates leads to the restoration of full deacetylase activity suggesting that inositol phosphates might be bona fide regulators of HDAC3 activity. The concentrations of these small signalling molecules in the cell has been shown to be sufficiently high to make them physiologically relevant regulators of HDAC activity (Barker et al. 2004). Whilst the exact mechanism of activation by inositol phosphate binding has yet to be fully determined there is some evidence that this involves the stabilisation of the active site channel (Watson et al. 2012; Arrar et al. 2013). The biological rationale for regulation of HDAC3 by inositol phosphates remains to be established.

6 The SMRT/NCoR Complex Is A Paradigm for Other Class I HDAC Complexes

Intriguingly, several other corepressor proteins that recruit HDACs 1&2 contain very similar SANT domains to that of the SMRT-DAD. These include the MTA proteins from the NuRD complex (nucleosome remodelling and deacetylase complex) and RCOR proteins from the CoREST complex (cofactor of REST). In both these corepressors, the SANT domain is preceded by an ELM2 domain that has been shown to be important for HDAC recruitment (Toh et al. 2000; Lee et al. 2006). Although, not as firmly established as for the SMRT/NCoR complex, there is evidence that both the NuRD and CoREST complexes associate with nuclear receptors. MTA1 directly binds estrogen receptor-α via an nuclear receptor binding motif found in a naturally occurring short form of the corepressor protein (Kumar et al. 2002). LSD1 (part of the CoREST complex) associates with the androgen receptor (Metzger et al. 2005).

The structure of MTA1 bound to HDAC1 shows that the MTA1-SANT domain binds to the HDAC1 in a very similar fashion to the SMRT-DAD domain binding to HDAC3 (Millard et al. 2013). As was observed for the HDAC3:SMRT complex, there is a basic inositol binding pocket formed at the interface between HDAC1 and the MTA1-SANT domain. Biochemical assays confirm that inositol phosphates also regulate the HDAC1 activity in this complex.

The ELM2 domain of MTA1 is also present in the HDAC1:MTA1 structure and is shown to wrap completely around the catalytic domain of HDAC1 in an extended groove. This positions the amino-terminus of the ELM2 domain and carboxy-terminus of the SANT domain on either side of the active site. Interaction studies with HDAC3 and SMRT show that, although there is only limited sequence conservation, a region amino-terminal to the SANT domain in SMRT also contributes to interaction with HDAC3 (Millard et al. 2013). This is likely to mimic the ELM2 domain, and so wrap around HDAC3, drawing TBL1 and GPS2 closer to the histone deacetylase. This extensive interface would correlate well with the observed stability of the HDAC3:SMRT complex.

HDACs 1&2 are highly similar (83 % identical) and are recruited interchangeably to the same repression complexes including the NuRD, CoREST and Sin3A complexes (Laherty et al. 1997; Xue et al. 1998; Lee et al. 2005). In contrast, whilst HDAC3 is also similar to HDACs 1&2 (53 % identical), it is recruited uniquely to the SMRT/NCoR complex. Careful comparison of the interactions of MTA1 and SMRT with HDACs 1&3, respectively, reveal a series of subtle but sufficient differences to result in the HDACs being recruited their cognate partners. In particular, there are two distinct regions in that contribute primarily to the specificity of assembling these highly related HDAC:corepressor complexes (Fig. 4) (Millard et al. 2013).

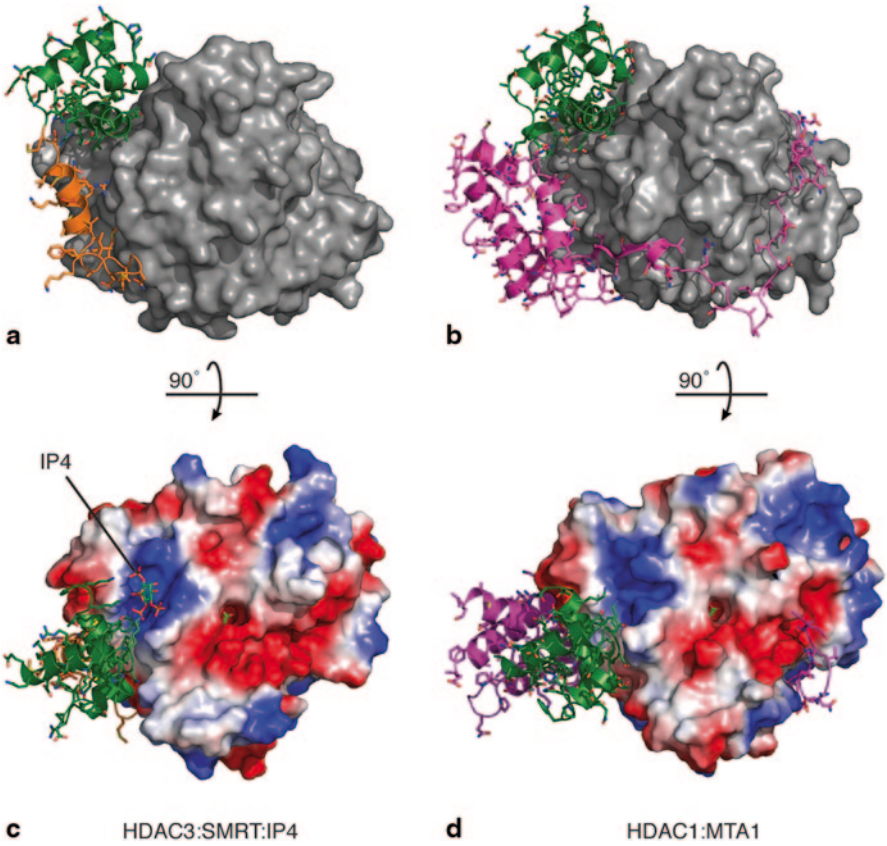


Fig. 4 Structures of **a** HDAC3:SMRT and **b** HDAC1:MTA1 corepressor complexes. The HDACs are illustrated as surfaces (*grey*) with the bound corepressors are shown as cartoons. These are coloured to highlight the SANT domains (*green*), helix H0 of SMRT (*orange*) and the ELM2 domain of MTA1 (*magenta*). The HDAC active sites are located at the *top* of each panel. Electrostatic surface profiles of **c** HDAC3 and **d** HDAC1 with their cognate corepressors following a 90° rotation. An acetate molecule can be seen in the HDAC active sites (*green*). Inositol phosphate (*green* and *orange*) is bound to the HDAC3:SMRT in a basic binding pocket at the interface between the molecules. A similar basic pocket is formed at the interface between HDAC1 and MTA1 and could accommodate an inositol phosphate molecule. HDAC3:SMRT (pdbcode 4A69) and HDAC1:MTA1 (pdbcode 4BKX)

7 Recruitment of Repression Complexes to Nuclear Receptors

Corepressor proteins are recruited to specific genomic loci through interactions with the ligand-binding domains (LBDs) of unliganded nuclear receptors. There are 48 unique receptors and many of their LBDs have been structurally characterised by crystallography (reviewed in (Rastinejad et al. 2013)). The LBD has a three-layered

α-helical sandwich fold and the ligand-binding pocket is found within the middle layer (Bourguet et al. 1995; Renaud et al. 1995; Wagner et al. 1995). The ligand is shielded from the external environment when bound within this hydrophobic pocket. A carboxy-terminal helix (known as helix 12 or AF2 helix) lies across this pocket and can make direct contact with the ligand.

Before exploring how repression complexes are recruited to nuclear receptors, it is useful to consider how coactivators are recruited to ligand-bound nuclear receptors. Sequence alignment of short activating fragments from RIP140, SRC1 and CBP identified a highly conserved consensus motif LxxLL (NR box) that was sufficient for binding (Heery et al. 1997; Darimont et al. 1998). The molecular detail of NR box recruitment has been revealed through structural studies of isolated LBDs bound to short coactivator peptides (Nolte et al. 1998; Watkins et al. 2003). The LxxLL motif adopts a helical structure on binding to the surface of the LBD and makes contact along a hydrophobic binding groove formed by helices 3, 4, 5 and 12. Helix 12 is promoted to the "active" conformation on ligand binding and is essential to support coactivator binding (reviewed in (Nagy and Schwabe 2004)).

More recently, full-length nuclear receptors have been characterised bound to coactivator peptides (Chandra et al. 2008; Chandra et al. 2013; Lou et al. 2014). The structures of full-length heterodimer PPARγ-RXRα, HNF4α homodimer, and RXRα-LXRβbound to DNA provide insight into the relative positioning of the DNA-binding domain with respect to the LBD. Peptide binding to the full-length receptor is largely similar to that seen in the isolated LBDs, and since binding is some distance from the DNA-binding domain, it is suggested that the other domains do not directly modulate coactivator binding.

In contrast to coactivator binding, repression complex recruitment to nuclear receptors is more favourable in the absence of ligand. Mapping studies and sequence alignment revealed that recruitment occurs through the consensus motif LxxH/IIxxxI/L (CoRNR box) (Hu and Lazar 1999; Nagy et al. 1999; Perissi et al. 1999). There are three CoRNR box motifs or interaction domains (ID1, ID2 and ID3) that occur in both SMRT and NCoR (Webb et al. 2000). The first structure of an LBD (PPARa bound to antagonist GW6471) with bound corepressor peptide ID1 (SMRT) showed that the CoRNR box assumes a helical fold and its binding prevents helix 12 from assuming an active conformation (Xu et al. 2002). This crystal structure showed that coactivator and corepressor binding is mutually exclusive as both bind to the same surface of the LBD. The SMRT corepressor peptide has a larger interaction interface with the LBD than that of coactivator motifs and is not dependant on helix 12. Further LBD crystal structures with bound corepressor peptides have been solved, and of note, are the two ligand-free corepressor-bound structures that are now available (structures and relevant references are detailed in Table 1). The first of these structures, Rev-erbα bound to ID2 (NCoR), showed that NCoR forms both the expected α-helix but also an unanticipated antiparallel β-sheet with helix 11 of the LBD (Phelan et al. 2010). This β-structure may be a feature that is specifically tailored for recruitment of Rev-erbα by ID2 (NCoR) but would not occur with ID1 (NCoR) due to sequence differences.

Table 1 A wide range of nuclear receptors have been structurally characterised with bound CoRNR box motifs (LxxH/IIxxxI/L) from NCoR and SMRT, and an NR box motif (LxxLL) from RIP140. Where indicated, the nuclear receptors have been crystallised in the presence of ligand. Numbering of CoRNR box motifs has been inconsistent but here the sites are numbered from the carboxy-terminus

Corepressor	Position	Residues	PDBcode	NR	Ligand	Reference
NCoR ID3	1933–1937	NFIDVIITRQIAS	–	–		–
NCoR ID2	2055–2059	DHICQIITQDFAR	3N00	Rev-erbα (NR1D1)	None	(Phelan et al. 2010)
			3KMZ	RARα (NR1B1)	Inverse agonist (BMS493)	(le Maire et al. 2010)
NCoR ID1	2263–2267	LGLEDIIRKALMG	2OVM	PR (NR3C3)	Antagonist (Asoprisnil)	(Madauss et al. 2007)
			3 H52	GR (NR3C1)	Antagonist (Mifepristone)	(Schoch et al. 2010)
			4II6	FXR (NR1H4)	Partial agonist (Ivermectin)	(Jin et al. 2013)
SMRT ID3[a]	Insert at 2049	TFIDAIIMRQIAH	–	–		–
SMRT ID2	2147–2151	QHISEVITQDYTR	–	–		–
SMRT ID1	2350–2354	MGLEAIIRKALMG	1KKQ	PPARα (NR1C1)	Antagonist (GW6471)	(Xu et al. 2002)
			2GPV	ERRγ (NR3B3)	Inverse agonist (4-OHT)	(Wang et al. 2006)
			2OVH	PR	Antagonist (Asoprisnil)	(Madauss et al. 2007)
			3R29	RXRα (NR2B1)	None	(Zhang et al. 2011)
			3R2A	RXRα	Antagonist (Rhein)	(Zhang et al. 2011)
RIP140 ID5	366–390	NSLLLHLLKS	2GPP	ERRγ	Agonist (GSK4716)	(Wang et al. 2006)
			2GPO	ERRγ	None	(Wang et al. 2006)

[a] SMRT ID3 is present in an alternatively spliced variant containing 38 additional amino acids and is found between exons 37 and 38 (Malartre et al. 2004)

The second of these structures, RXRα bound to ID1 (SMRT), was solved with the nuclear receptor in both the unliganded and antagonist bound state. The unliganded receptor was shown to be a tetramer in the crystal structure with each monomer binding to one SMRT peptide (Zhang et al. 2011). Interestingly the antagonist displaced the SMRT peptide allowing helix 12 from neighbouring molecules to bind to the vacated corepressor binding site.

Much work has focused on understanding how the LBD can switch binding preference from coactivator to corepressor. Several lines of evidence suggest that ligand binding promotes the stabilisation of the LBD, and this stabilisation drives coactivator binding, rather than the absolute position of helix 12 relative to the rest of the domain. Examination of the crystallography temperature factors of apo-LBDs, and further NMR mobility studies, suggest that the lower ligand-binding section of the domain is more mobile that the upper portion of the LBD (Nolte et al. 1998; Cronet et al. 2001; Watkins et al. 2003). Further biochemical studies suggest that ligand binding stabilises the receptor, and this causes the LBD to become more compact and rigid (Keidel et al. 1994; Pissios et al. 2000). In the absence of ligand, the longer CoRNR box motif stabilises the ligand-free LBD. The mobility of helix 12 itself has been studied by fluorescence anisotropy and the helix shows much slower dynamics on ligand binding, suggesting that it associates with the surface of the LBD, presumably in the active position (Kallenberger et al. 2003). Therefore, helix 12 acts as a readout of ligand state, and plays a key role in selectively recruiting either coactivator or corepressor.

In contrast to SMRT and NCoR, RIP140 and LCoR are corepressor proteins that associate with ligand-bound nuclear receptors by means of an LxxLL motif. This motif occurs nine times in RIP140 and a single motif has been identified in LCoR (Heery et al. 1997; Fernandes et al. 2003). The recruitment of corepressors through the LxxLL motif suggests a very different biological rationale for repression since this sequence is most commonly found in coactivator proteins and mediates their recruitment to nuclear receptors (Cavaillès et al. 1995). However, like SMRT and NCoR, RIP140 and LCoR act as corepressors through the recruitment of HDACs complexes (Wei et al. 2000; Fernandes et al. 2003).

The stoichiometry of corepressor binding to dimeric, DNA bound nuclear receptors remains to be fully established. SMRT and other corepressors contain more than one NR or CoRNR box motif and therefore a single corepressor could in principle interact with both nuclear receptors in a homo- or hetero-dimer. Interestingly, several coregulator complexes are dimeric and contain two corepressor proteins that could both make interactions with nuclear receptor ligand binding domains. Although we can only speculate about the assembly of corepressor complexes, recent work has shown that the coactivator PGC-1α is recruited to ERRα and ERRγ asymmetrically such that a single NR box motif efficiently interacts with just one subunit of the homodimeric receptor (Takacs et al. 2013).

8 Recruitment of Other Transcriptional Regulators

SMRT and NCoR have been shown to associate with numerous other proteins beside those already described. In general, these further interactions tend to be relatively transient in comparison, but are nevertheless specific. Some of these interactions have only been loosely mapped to extended regions within SMRT, but others have been mapped in detail, and some of these interaction sites have been characterised through structural studies.

A small peptide from SMRT has been crystallised with the repressive transcription factor BCL6 and is shown to adopt a beta-strand on binding (Ahmad et al. 2003). BCL6 is required for normal B cell maturation, and deregulated expression leads to B-cell non-Hodgkin lymphomas. Residues 1414–1430 of SMRT bind to the homodimeric BTB domain of BCL6, with the amino-terminus of the SMRT peptide contributing a β-strand along an existing β-sheet at the bottom of the dimer, while the rest of the peptide binds in an extended conformation on the surface of the domain. BCL6 has also been crystallised in complex with a 17 residue peptide from BCoR (a non-homologous corepressor), and although it does not show any significant sequence similarity with the SMRT peptide, both peptides bind along the same lateral groove in BCL6 (Ghetu et al. 2008).

Fluorescently labelled peptides were used to accurately map a binding region of SMRT to the chimeric protein AML1/ETO (Gelmetti et al. 1998). AML1/ETO acts as a transcription regulator that represses proliferation and differentiation of primary bone marrow cells through an interaction with SMRT. The AML1/ETO chimera is formed through a chromosomal translocation and has been shown to cause acute myeloid leukemia. The NMR structure of AML1/ETO bound to residues 1101–1113 of SMRT showed that the SMRT peptide forms a short antiparallel β-sheet on the surface of the MYND domain, and the rest of the peptide binds in an extended conformation in a hydrophobic pocket of the domain (Liu et al. 2007).

The transcriptional regulator SHARP binds to SMRT at its very carboxy-terminus (residues 2257–2517) through a conserved acidic (LSD) motif (Ariyoshi and Schwabe 2003). The SMRT peptide requires phosphorylation on the serine residue of this motif in order to show increased binding affinity to SHARP (Mikami et al. 2014). Several other transcriptional regulators have been shown to bind to SMRT including DACH1, DEAF1 and Kaiso (Yoon et al. 2003; Wu et al. 2003; Kateb et al. 2013). In addition to HDAC3, other histone deacetylase enzymes are recruited by SMRT. Interactions between SMRT with HDAC1, HDAC4, HDAC5, HDAC7 and Sirt1 have been reported (Nagy et al. 1997; Kao et al. 2000; Fischle et al. 2001, 2002; Picard et al. 2004). Furthermore, the SMRT complex has been shown to interact with other chromatin modifying enzymes, such as the histone demethylase JMJD2A (Zhang et al. 2005). The structural details of these complexes remain to be determined.

9 Chromatin Targeting Through Corepressor Associated Proteins

It is firmly established that the SMRT complex is targeted to specific genomic loci through interactions with transcription factors such as nuclear receptors that have their own DNA binding activity. However, targeting of repression complexes can be achieved through associated chromatin targeting domains. The only intrinsic chromatin-targeting domain within SMRT/NCoR identified so far is the SANT2 domain (Yu et al. 2003). Several other chromatin interacting proteins contain SANT domains, including those from Ada2 and ISWI, share the property of histone binding (Boyer et al. 2002; Grüne et al. 2003).

Further chromatin targeting of the SMRT repression complex may occur through the WD40 domains of TBL1. Corepressor proteins such as RBBP7 contain the WD40 domain fold and have been shown to bind to histone tails (Murzina et al. 2008). There are four WD40 domains within each SMRT complex since TBL1 tetramerises, and it is possible that either each domain could bind to histone tails from the same stretch of chromatin, or alternatively could draw distant chromatin strands closer together.

Recruitment to chromatin through coregulator-associated proteins has been shown to be a sequential and regulated process. The first study to identify the ordered recruitment of coregulator complexes followed the transcriptional activation of the HO gene in budding yeast (Cosma et al. 1999). They found that transcription factors, coactivator and corepressor complexes arrived and left in a set pattern that was precisely timed. Similar scenarios have been suggested for corepressor recruitment in higher eukaryotes, and in one study, ligand bound ERα was shown to sequentially recruit the SMRT and the NURD complex (Liu and Bagchi 2004). A more detailed account of ordered recruitment can be found in the following review (Perissi et al. 2010).

10 Post-transcriptional Modifications Influencing Corepressor Function

Both SMRT and NCoR are subject to extensive alternative mRNA splicing and this regulates their activity (Goodson et al. 2005). Splicing has been shown to occur *in vivo* to generate multiple SMRT and NCoR variants that have distinguishable repression characteristics. SMRT can be alternatively spliced to contain either one, two or three CoRNR box motifs and this has a pronounced effect during differentiation and development (Short et al. 2005; Malartre et al. 2006; Goodson et al. 2011). The multiple isoforms of SMRT and NCoR allow diversified roles for the various splice variants, presumably through the recruitment of different interacting partners.

Post-translational modification of SMRT and NCoR, such as phosphorylation and ubiquitination, create alternative binding surfaces for interaction partners. In

some cases these modifications are added sequentially to bring about the desired outcome. For example, SMRT is targeted by 14-3-3ε and UBE2D1 following phosphorylation by IKKα at residue Ser2410 (Hoberg et al. 2004). This targets SMRT for export from the nucleus, followed by proteosomal degradation. The ubiquitin ligase Siah2 is specific for NCoR, but not for SMRT. Siah2 is an E3 ligase that interacts with UBE2E2 and effectively reverses NCoR-mediated transcriptional repression through ubiquitination (Zhang et al. 1998). Phosphorylation of SMRT by casein kinase 2 on Ser1492 stabilises the association between SMRT and nuclear receptors, thus enhancing repression (Zhou et al. 2001). Another SMRT specific degradation pathway has also been identified and involves the peptidyl-prolyl isomerase Pin1 (Stanya et al. 2008). It is likely that modifications such as acetylation, SUMOylation and methylation of residues within SMRT and NCoR will modulate their activity and will lead to further diversification of their properties.

11 Conclusions

Corepressors are large scaffold proteins whose essential role is to recruit chromatin modifying enzymes to the genome so as to bring about transcriptional repression. Many functional and structural studies are beginning to clarify to the molecular details of protein-protein interactions within these complexes. The SMRT and NCoR repression complexes are the best-characterised nuclear receptor corepressors. They assemble a stable multivalent core complex containing a tetramer of TBL1X and two copies of HDAC3, GPS2 and SMRT or NCoR. Beyond the core complex the corepressors are largely disordered, but contain many short sequence motifs essential for forming transient interactions with transcription factors (including unliganded nuclear receptors) and other proteins that contribute to transcription repression.

The recent finding that HDAC activity in the complex is dependent upon an associated inositol phosphate molecule raises the intriguing question as to whether transcriptional repression may be regulated by this small molecule. It remains to be established whether the levels of inositol phosphates are regulated in individual compartments of the cell, perhaps during the cell cycle or in a circadian fashion.

Acknowledgements We thank Louise Fairall for critical reading of the manuscript. This work was supported by the Wellcome Trust (grant number WT085408) and the BBSRC (grant number 10867).

References

Ahmad KF, Melnick A, Lax S et al (2003) Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. Mol Cell 12:1551–1564

Ariyoshi M, Schwabe JWR (2003) A conserved structural motif reveals the essential transcriptional repression function of Spen proteins and their role in developmental signaling. Genes Dev 17:1909–1920. doi:10.1101/gad.266203

Arrar M, Turnham R, Pierce L et al (2013) Structural insight into the separate roles of inositol tetraphosphate and deacetylase-activating domain in activation of histone deacetylase 3. Protein Sci 22:83–92. doi:10.1002/pro.2190

Barker CJ, Wright J, Hughes PJ et al (2004) Complex changes in cellular inositol phosphate complement accompany transit through the cell cycle. Biochem J 380:465–473. doi:10.1042/BJ20031872

Bassi MT, Ramesar RS, Caciotti B et al (1999) X-linked late-onset sensorineural deafness caused by a deletion involving OA1 and a novel gene containing WD-40 repeats. Am J Hum Genet 64:1604–1616. doi:10.1086/302408

Bourguet W, Ruff M, Chambon P et al (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. Nature 375:377–382. doi:10.1038/375377a0

Boyer LA, Langer MR, Crowley KA et al (2002) Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. Mol Cell 10:935–942

Boyer LA, Latek RR, Peterson CL (2004) The SANT domain: a unique histone-tail-binding module? Nat Rev Mol Cell Biol 5:158–163. doi:10.1038/nrm1314

Cavaillès V, Dauvois S, L'Horset F et al (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J 14:3741–3751

Chandra V, Huang P, Hamuro Y et al (2008) Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. Nature 456:350–356. doi:10.1038/nature07413

Chandra V, Huang P, Potluri N et al (2013) Multidomain integration in the structure of the HNF-4a nuclear receptor complex. Nature 495:394–398. doi:10.1038/nature11966

Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457. doi:10.1038/377454a0

Codina A, Love JD, Li Y et al (2005) Structural insights into the interaction and activation of histone deacetylase 3 by nuclear receptor corepressors. Proc Natl Acad Sci U S A 102:6009–6014. doi:10.1073/pnas.0500299102

Cohen RN, Brzostek S, Kim B et al (2001) The specificity of interactions between nuclear hormone receptors and corepressors is mediated by distinct amino acid sequences within the interacting domains. Mol Endocrinol 15:1049–1061. doi:10.1210/mend.15.7.0669

Cosma MP, Tanaka T, Nasmyth K (1999) Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell 97:299–311

Couture J-F, Collazo E, Trievel RC (2006) Molecular recognition of histone H3 by the WD40 protein WDR5. Nat Struct Mol Biol 13:698–703. doi:10.1038/nsmb1116

Cronet P, Petersen JF, Folmer R et al (2001) Structure of the PPARalpha and -gamma ligand binding domain in complex with AZ 242; ligand selectivity and agonist activation in the PPAR family. Structure 9:699–706

Dangond F, Hafler DA, Tong JK et al (1998) Differential display cloning of a novel human histone deacetylase (HDAC3) cDNA from PHA-activated immune cells. Biochem Biophys Res Commun 242:648–652. doi:10.1006/bbrc.1997.8033

Darimont BD, Wagner RL, Apriletti JW et al (1998) Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 12:3343–3356

Doyon Y, Cayrou C, Ullah M et al (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol Cell 21:51–64. doi:10.1016/j.molcel.2005.12.007

Fernandes I, Bastien Y, Wai T et al (2003) Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. Mol Cell 11:139–150

Finnin MS, Donigian JR, Cohen A et al (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 401:188–193. doi:10.1038/43710

Fischle W, Dequiedt F, Fillion M et al (2001) Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J Biol Chem 276:35826–35835. doi:10.1074/jbc.M104935200

Fischle W, Dequiedt F, Hendzel MJ et al (2002) Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell 9:45–57

Gelmetti V, Zhang J, Fanelli M et al (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol 18:7185–7191

Ghetu AF, Corcoran CM, Cerchietti L et al (2008) Structure of a BCOR corepressor peptide in complex with the BCL6 BTB domain dimer. Mol Cell 29:384–391. doi:10.1016/j.molcel.2007.12.026

Goodson M, Jonas BA, Privalsky MA (2005) Corepressors: custom tailoring and alterations while you wait. Nucl Recept Signal 3:e003. doi:10.1621/nrs.03003

Goodson ML, Mengeling BJ, Jonas BA, Privalsky ML (2011) Alternative mRNA splicing of corepressors generates variants that play opposing roles in adipocyte differentiation. J Biol Chem 286:44988–44999. doi:10.1074/jbc.M111.291625

Grüne T, Brzeski J, Eberharter A et al (2003) Crystal structure and functional analysis of a nucleosome recognition module of the remodeling factor ISWI. Mol Cell 12:449–460

Guenther MG, Lane WS, Fischle W et al (2000) A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. Genes Dev 14:1048–1057

Guenther MG, Barak O, Lazar MA (2001) The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol Cell Biol 21:6091–6101

Hartman HB, Yu J, Alenghat T et al (2005) The histone-binding code of nuclear receptor corepressors matches the substrate specificity of histone deacetylase 3. EMBO Rep 6:445–451. doi:10.1038/sj.embor.7400391

Heery DM, Kalkhoven E, Hoare S, Parker MG (1997) A signature motif in transcriptional coactivators mediates binding to nuclear receptors. Nature 387:733–736. doi:10.1038/42750

Hoberg JE, Yeung F, Mayo MW (2004) SMRT derepression by the IkappaB kinase alpha: a prerequisite to NF-kappaB transcription and survival. Mol Cell 16:245–255. doi:10.1016/j.molcel.2004.10.010

Hörlein AJ, Näär AM, Heinzel T et al (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404. doi:10.1038/377397a0

Hu X, Lazar MA (1999) The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402:93–96. doi:10.1038/47069

Jepsen K, Hermanson O, Onami TM et al (2000) Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell 102:753–763

Jepsen K, Solum D, Zhou T et al (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 450:415–419. doi:10.1038/nature06270

Jin L, Feng X, Rong H et al (2013) The antiparasitic drug ivermectin is a novel FXR ligand that regulates metabolism. Nat Commun 4:1937. doi:10.1038/ncomms2924

Kallenberger BC, Love JD, Chatterjee VKK, Schwabe JWR (2003) A dynamic mechanism of nuclear receptor activation and its perturbation in a human disease. Nat Struct Biol 10:136–140. doi:10.1038/nsb892

Kao HY, Downes M, Ordentlich P, Evans RM (2000) Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev 14:55–66

Kateb F, Perrin H, Tripsianes K et al (2013) Structural and functional analysis of the DEAF-1 and BS69 MYND domains. PLoS One 8:e54715. doi:10.1371/journal.pone.0054715

Keidel S, LeMotte P, Apfel C (1994) Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. Mol Cell Biol 14:287–298

Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705. doi:10.1016/j.cell.2007.02.005

Kulozik P, Jones A, Mattijssen F et al (2011) Hepatic deficiency in transcriptional cofactor TBL1 promotes liver steatosis and hypertriglyceridemia. Cell Metab 13:389–400. doi:10.1016/j.cmet.2011.02.011

Kumar R, Wang R-A, Mazumdar A et al (2002) A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. Nature 418:654–657. doi:10.1038/nature00889

Laherty CD, Yang WM, Sun JM et al (1997) Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell 89:349–356
le Maire A, Teyssier C, Erb C et al (2010) A unique secondary-structure switch controls constitutive gene repression by retinoic acid receptor. Nat Struct Mol Biol 17:801–807. doi:10.1038/nsmb.1855
Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437:432–435. doi:10.1038/nature04021
Lee MG, Wynder C, Bochar DA et al (2006) Functional interplay between histone demethylase and deacetylase enzymes. Mol Cell Biol 26:6395–6402. doi:10.1128/MCB.00723-06
Li J, Wang J, Wang J et al (2000) Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J 19:4342–4350. doi:10.1093/emboj/19.16.4342
Liu X-F, Bagchi MK (2004) Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters in vivo. J Biol Chem 279:15050–15058. doi:10.1074/jbc.M311932200
Liu Y, Chen W, Gaudet J et al (2007) Structural basis for recognition of SMRT/N-CoR by the MYND domain and its contribution to AML1/ETO's activity. Cancer Cell 11:483–497. doi:10.1016/j.ccr.2007.04.010
Lonard DM, O'Malley BW (2007) Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. Mol Cell 27:691–700. doi:10.1016/j.molcel.2007.08.012
Lou X, Toresson G, Benod C et al (2014) Structure of the retinoid X receptor a-liver X receptor b (RXRa-LXRb) heterodimer on DNA. Nat Struct Mol Biol. doi:10.1038/nsmb.2778
Madauss KP, Grygielko ET, Deng S-J et al (2007) A structural and in vitro characterization of asoprisnil: a selective progesterone receptor modulator. Mol Endocrinol 21:1066–1081. doi:10.1210/me.2006–0524
Makowski A, Brzostek S, Cohen RN, Hollenberg AN (2003) Determination of nuclear receptor corepressor interactions with the thyroid hormone receptor. Mol Endocrinol 17:273–286. doi:10.1210/me.2002–0310
Malartre M, Short S, Sharpe C (2004) Alternative splicing generates multiple SMRT transcripts encoding conserved repressor domains linked to variable transcription factor interaction domains. Nucleic Acids Res 32:4676–4686. doi:10.1093/nar/gkh786
Malartre M, Short S, Sharpe C (2006) Xenopus embryos lacking specific isoforms of the corepressor SMRT develop abnormal heads. Dev Biol 292:333–343. doi:10.1016/j.ydbio.2006.01.007
Mathur M, Tucker PW, Samuels HH (2001) PSF is a novel corepressor that mediates its effect through Sin3A and the DNA binding domain of nuclear hormone receptors. Mol Cell Biol 21:2298–2311. doi:10.1128/MCB.21.7.2298–2311.2001
McKenna NJ, Lanz RB, O'Malley BW (1999) Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20:321–344
Métivier R, Penot G, Hübner MR et al (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115:751–763
Metzger E, Wissmann M, Yin N et al (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436–439. doi:10.1038/nature04020
Mikami S, Kanaba T, Takizawa N et al (2014) Structural insights into the recruitment of SMRT by the corepressor SHARP under phosphorylative regulation. Structure 22:35–46. doi:10.1016/j.str.2013.10.007
Millard CJ, Watson PJ, Celardo I et al (2013) Class I HDACs share a common mechanism of regulation by inositol phosphates. Mol Cell 51:57–67. doi:10.1016/j.molcel.2013.05.020
Murzina NV, Pei X-Y, Zhang W et al (2008) Structural basis for the recognition of histone H4 by the histone-chaperone RbAp46. Structure 16:1077–1085. doi:10.1016/j.str.2008.05.006
Musselman CA, Lalonde M-E, Côté J, Kutateladze TG (2012) Perceiving the epigenetic landscape through histone readers. Nat Struct Mol Biol 19:1218–1227. doi:10.1038/nsmb.2436
Nagy L, Schwabe JWR (2004) Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci 29:317–324. doi:10.1016/j.tibs.2004.04.006

Nagy L, Kao HY, Chakravarti D et al (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 89:373–380

Nagy L, Kao HY, Love JD et al (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev 13:3209–3216

Nolte RT, Wisely GB, Westin S et al (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature 395:137–143. doi:10.1038/25931

Oberoi J, Fairall L, Watson PJ et al (2011) Structural basis for the assembly of the SMRT/NCoR core transcriptional repression machinery. Nat Struct Mol Biol 18:177–184. doi:10.1038/nsmb.1983

Perissi V, Staszewski LM, McInerney EM et al (1999) Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev 13:3198–3208

Perissi V, Jepsen K, Glass CK, Rosenfeld MG (2010) Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet 11:109–123. doi:10.1038/nrg2736

Phelan CA, Gampe RT, Lambert MH et al (2010) Structure of Rev-erbalpha bound to N-CoR reveals a unique mechanism of nuclear receptor-co-repressor interaction. Nat Struct Mol Biol 17:808–814. doi:10.1038/nsmb.1860

Picard F, Kurtev M, Chung N et al (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 429:771–776. doi:10.1038/nature02583

Pissios P, Tzameli I, Kushner P, Moore DD (2000) Dynamic stabilization of nuclear receptor ligand binding domains by hormone or corepressor binding. Mol Cell 6:245–253

Potter GB, Potter GB, Beaudoin GM et al (2001) The hairless gene mutated in congenital hair loss disorders encodes a novel nuclear receptor corepressor. Genes Dev 15:2687–2701. doi:10.1101/gad.916701

Pugh TJ, Weeraratne SD, Archer TC et al (2012) Medulloblastoma exome sequencing uncovers subtype-specific somatic mutations. Nature 488:106–110. doi:10.1038/nature11329

Qin S, Parthun MR (2006) Recruitment of the type B histone acetyltransferase Hat1p to chromatin is linked to DNA double-strand breaks. Mol Cell Biol 26:3649–3658. doi:10.1128/MCB.26.9.3649–3658.2006

Rastinejad F, Huang P, Chandra V, Khorasanizadeh S (2013) Understanding nuclear receptor form and function using structural biology. J Mol Endocrinol 51:T1–21. doi:10.1530/JME-13-0173

Renaud JP, Rochel N, Ruff M et al (1995) Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. Nature 378:681–689. doi:10.1038/378681a0

Ruthenburg AJ, Wang W, Graybosch DM et al (2006) Histone H3 recognition and presentation by the WDR5 module of the MLL1 complex. Nat Struct Mol Biol 13:704–712. doi:10.1038/nsmb1119

Ruthenburg AJ, Allis CD, Wysocka J (2007) Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. Mol Cell 25:15–30. doi:10.1016/j.molcel.2006.12.014

Santos GM, Fairall L, Schwabe JWR (2011) Negative regulation by nuclear receptors: a plethora of mechanisms. Trends Endocrinol Metab 22:87–93. doi:10.1016/j.tem.2010.11.004

Sanyal S, Båvner A, Haroniti A et al (2007) Involvement of corepressor complex subunit GPS2 in transcriptional pathways governing human bile acid biosynthesis. Proc Natl Acad Sci U S A 104:15665–15670. doi:10.1073/pnas.0706736104

Schoch GA, D'Arcy B, Stihle M et al (2010) Molecular switch in the glucocorticoid receptor: active and passive antagonist conformations. J Mol Biol 395:568–577. doi:10.1016/j.jmb.2009.11.011

Short S, Malartre M, Sharpe C (2005) SMRT has tissue-specific isoform profiles that include a form containing one CoRNR box. Biochem Biophys Res Commun 334:845–852. doi:10.1016/j.bbrc.2005.06.175

Spain BH, Bowdish KS, Pacal AR et al (1996) Two human cDNAs, including a homolog of Arabidopsis FUS6 (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammalian cells. Mol Cell Biol 16:6698–6706

Stanya KJ, Liu Y, Means AR, Kao H-Y (2008) Cdk2 and Pin1 negatively regulate the transcriptional corepressor SMRT. J Cell Biol 183:49–61. doi:10.1083/jcb.200806172

Tagami T, Park Y, Jameson JL (1999) Mechanisms that mediate negative regulation of the thyroid-stimulating hormone alpha gene by the thyroid hormone receptor. J Biol Chem 274:22345–22353
Takacs M, Petoukhov MV, Atkinson RA et al (2013) The asymmetric binding of PGC-1a to the ERRa and ERRg nuclear receptor homodimers involves a similar recognition mechanism. PLoS One 8:e67810. doi:10.1371/journal.pone.0067810
Taunton J, Hassig CA, Schreiber SL (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272:408–411
Toh Y, Kuninaka S, Endo K et al (2000) Molecular analysis of a candidate metastasis-associated gene, MTA1: possible interaction with histone deacetylase 1. J Exp Clin Cancer Res 19:105–111
Toubal A, Clément K, Fan R et al (2013) SMRT-GPS2 corepressor pathway dysregulation coincides with obesity-linked adipocyte inflammation. J Clin Invest 123:362–379. doi:10.1172/JCI64052
Turner BM (1993) Decoding the nucleosome. Cell 75:5–8
Varlakhanova N, Hahm JB, Privalsky ML (2011) Regulation of SMRT corepressor dimerization and composition by MAP kinase phosphorylation. Mol Cell Endocrinol 332:180–188. doi:10.1016/j.mce.2010.10.010
Wagner RL, Apriletti JW, McGrath ME et al (1995) A structural role for hormone in the thyroid hormone receptor. Nature 378:690–697. doi:10.1038/378690a0
Wang L, Zuercher WJ, Consler TG et al (2006) X-ray crystal structures of the estrogen-related receptor-gamma ligand binding domain in three functional states reveal the molecular basis of small molecule regulation. J Biol Chem 281:37773–37781. doi:10.1074/jbc.M608410200
Wang Z, Zang C, Cui K et al (2009) Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell 138:1019–1031. doi:10.1016/j.cell.2009.06.049
Watkins RE, Davis-Searles PR, Lambert MH, Redinbo MR (2003) Coactivator binding promotes the specific interaction between ligand and the pregnane X receptor. J Mol Biol 331:815–828
Watson PJ, Fairall L, Santos GM, Schwabe JWR (2012) Structure of HDAC3 bound to co-repressor and inositol tetraphosphate. Nature 481:335–340. doi:10.1038/nature10728
Webb P, Anderson CM, Valentine C et al (2000) The nuclear receptor corepressor (N-CoR) contains three isoleucine motifs (I/LXXII) that serve as receptor interaction domains (IDs). Mol Endocrinol 14:1976–1985
Wei LN, Hu X, Chandra D et al (2000) Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. J Biol Chem 275:40782–40787. doi:10.1074/jbc.M004821200
Wen YD, Perissi V, Staszewski LM et al (2000) The histone deacetylase-3 complex contains nuclear receptor corepressors. Proc Natl Acad Sci U S A 97:7202–7207
Wu K, Yang Y, Wang C et al (2003) DACH1 inhibits transforming growth factor-beta signaling through binding Smad4. J Biol Chem 278:51673–51684. doi:10.1074/jbc.M310021200
Wysocka J, Swigut T, Milne TA et al (2005) WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development. Cell 121:859–872. doi:10.1016/j.cell.2005.03.036
Xu HE, Stanley TB, Montana VG et al (2002) Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARalpha. Nature 415:813–817. doi:10.1038/415813a
Xue Y, Wong J, Moreno GT et al (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell 2:851–861
Yang WM, Yao YL, Sun JM et al (1997) Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. J Biol Chem 272:28001–28007
Yoon H-G, Chan DW, Reynolds AB et al (2003) N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 12:723–734
Yu J, Li Y, Ishizuka T et al (2003) A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. EMBO J 22:3403–3410. doi:10.1093/emboj/cdg326
Zhang J, Guenther MG, Carthew RW, Lazar MA (1998) Proteasomal regulation of nuclear receptor corepressor-mediated repression. Genes Dev 12:1775–1780
Zhang J, Kalkum M, Chait BT, Roeder RG (2002) The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2. Mol Cell 9:611–623

Zhang D, Yoon H-G, Wong J (2005) JMJD2A is a novel N-CoR-interacting protein and is involved in repression of the human transcription factor achaete scute-like homologue 2 (ASCL2/Hash2). Mol Cell Biol 25:6404–6414. doi:10.1128/MCB.25.15.6404–6414.2005

Zhang H, Chen L, Chen J et al (2011) Structural basis for retinoic X receptor repression on the tetramer. J Biol Chem 286:24593–24598. doi:10.1074/jbc.M111.245498

Zhou Y, Gross W, Hong SH, Privalsky ML (2001) The SMRT corepressor is a target of phosphorylation by protein kinase CK2 (casein kinase II). Mol Cell Biochem 220:1–13

Part III
Taking Nuclear Receptor Structure into the Clinic

Thinking Outside the Box: Alternative Binding Sites in the Ligand Binding Domain of Nuclear Receptors

Nerea Gallastegui and Eva Estébanez-Perpiñá

1 Introduction

Nuclear receptors (NRs) are members of a large superfamily of evolutionary related ligand-dependent transcription factors that orchestrate the regulation of target gene expression. These DNA-binding proteins undergo changes in their conformation and dynamic behavior upon the binding of an endogenous ligand that in turn regulates the recruitment of coregulators and chromatin modifying machineries. It is therefore not surprising that NRs are considered key players in a broad spectrum of physiological phenomena, such as cell proliferation, metabolism and homeostasis. These characteristics make NRs prime targets for a wide range of diseases including cancer and metabolic diseases. The scientific focus for drug discovery in NRs has brought forward many important drugs currently in the clinic for several types of cancer such as tamoxifen (breast cancer), bicalutamide (BIC) and recently enzalutamide (ENZ, MDV3100) (both for prostate cancer (PCa)) (Singh et al. 2006; Harzstark and Small 2010; Haendler and Cleve 2012). Nevertheless, therapeutic strategies have almost exclusively focused in targeting their cocooned ligand-binding pocket (LBP), which is located in the heart of the domain known as the ligand binding domain (LBD), as it has been reiteratively proven that antagonist binding at this site prevents or induces alternative conformational changes incompatible with coactivator binding. However, although initially effective at blocking tumor growth, prolonged treatments with LBP-based treatments eventually fail, leading to unresponsiveness and inevitably tumor progression (Huggins 1967; Knudsen and Penning 2010; Haendler and Cleve 2012; Heinlein and Chang 2004; Bohl et al. 2005; Mohler et al. 2012; Yuan et al. 2013). Mutations in the LBP of NRs are one of the mechanisms underlying the development of drugs resistance to LBP-based drugs, which result in hypothesized conformational changes translated into functional implications were

E. Estébanez-Perpiñá (✉) · N. Gallastegui
Department of Biochemistry and Molecular Biology, Institute of Biomedicine of the University of Barcelona (IBUB), University of Barcelona (UB), Baldiri-Reixac 15–21, Barcelona 08028, Spain
e-mail: evaestebanez@ub.edu

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_10

antagonists behave as agonists. An excellent example is that of BIC, an FDA approved drug targeting the androgen receptor (AR/NR3C4) LBP in PCa, which is one of the most widely used AR antagonists, however PCa patients that received a prolonged treatment with BIC develop resistance to this drug and the treatment eventually exacerbate cancer growth (Masiello et al. 2002). The molecular mechanisms leading to BIC conversion from antagonist to agonist was reported to be caused by certain single mutations in the LBP such as that of W741L and W741C, which aid in the accommodation of this antiandrogen in an agonist conformation (Bohl et al. 2005). The second-generation antiandrogen ENZ maintains its efficacious clinical activity against some AR mutants that render other clinical drugs ineffective, such as W741L, and has been recently FDA approved for castration resistant PCa (CRPC). However in these last 2 years, cell lines resistant to ENZ and also the highly-related ARN-509, containing a different LBP mutation have been characterized (e.g. F876L) (Joseph et al. 2013; Korpal et al. 2013).

Therefore, due to the hurdle posed by the high number of mutations encountered in the LBP underlying drug resistance to current clinical antagonists, different alternative binding sites on the LBD of NRs have been proposed and are currently being addressed as possible alternative druggable sites that could be used in substitution of the LBP or most likely in combination with current drugs to overcome deleterious side effects and resistance. Furthermore, the studies of these sites have not only led to the opening of new ways of controlling NR actions in certain pathological pathways but also aided in a better basic understanding of the protein-protein interaction of different coregulators with the LBD.

2 The Ligand Binding Domain and Its Off-LBP Alternative Sites

Most NRs feature a LBD domain with a general fold comprising of 12 α-helices (H1–12) and 1–2 β-sheets forming a three-layer sandwich-like structure with a central-hidden LBP, which is the target of the cognate hormone or ligand. The most C-terminal helix is H12, which exhibits conformational versatility, upon binding of agonistic ligands; H12 is repositioned in a “mouse trap” like fashion, completing the LBP (Shiau et al. 1998; Nagy and Schwabe 2004; Togashi et al. 2005). This closing of the mouse trap shapes/completes an alternative binding site known as the coactivator binding surface or the AF-2 pocket which is formed mainly by H3, H4–H5 and H12 (Fig. 1) (Hur et al. 2004; Estébanez-Perpiñá et al. 2005a) and it is highly conserved among this superfamily of transcription factors. The hydrophobic and solvent-exposed AF-2 pocket of NRs interacts intimately with physiological coactivators through specific biomotifs called the “NR boxes”, such as LxxLL present in p160 coactivator protein family (where L is leucine and X is any amino acid). These motifs bind as amphipatic α-helices with the hydrophobic L residues arranging themselves into three hydrophobic sub-pockets found in the AF-2 formed by H5/H12, H3/H12 and H5/H3 respectively. Additionally, two clusters of charged residues on the LBD surface flank this hydrophobic groove and assist in the orientation

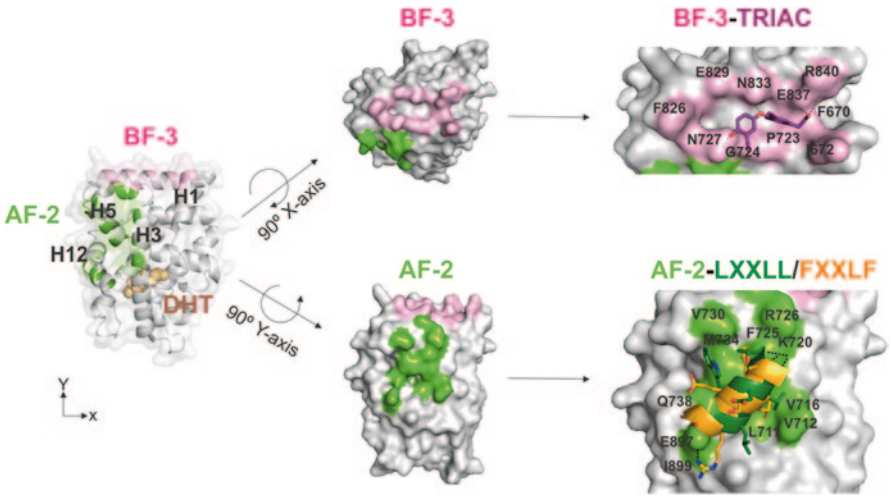


Fig. 1 The AR-LBD used as an example to represent location of alternative binding sites of NRs. Standard view of AR-LBD followed by −90° turn on x-axis and +90° turn on the y-axis showing both the BF-3 and the AF-2 pockets respectively. These two alternative sites are then showed in detail complexed to small molecule TRIAC as well as a LxxLL/FxxFF motif peptide. AF-2 and BF-3 site are shown in *green* and *pink* respectively first as a cartoon representation and then as a surface representation. The hormone DHT is depicted in brown as spheres. The TRIAC molecule (depicted in purple) is shown in stick form while the LxxLL/FxxFF peptide (*light green* and *orange*, respectively) is shown as a cartoon representation with the side chains in stick form. Hydrogen bond interactions of the molecules with AR are shown in black dotted lines

of the peptide motif, functioning as helix capping residues forming electrostatic interactions, known as a "charge clamp" and stabilizing the binding interactions. Furthermore, mutations in these charge clamp residues and other residues lining the AF-2 pocket have been implicated in pathology. Additionally, further sequences beyond the conserved hydrophobic motif help to confer specificity to the receptor-coactivator recognition. However, even though different NRs may bind to the same sets of coactivators, some NR AF-2 pockets exhibit a higher preference for certain NR boxes above others and the LxxLL flanking regions may also aid in their higher specificity. An excellent example is that of steroidal receptor coactivator 1 (SRC1/NCOA1), which contains 3 LxxLL NR boxes. While, the NRs thyroid receptors (TR/NR1A1 and NR1A2) and estrogen receptors (ER/NR3A1 and NR3A2) have been shown to bind with a higher affinity to box2, AR binds stronger to box3 (Northrop et al. 2000; Estébanez-Perpiñá et al. 2005b). Moreover, TR was shown to require two NR boxes (box2 and box3) for a higher affinity (Northrop et al. 2000).

The LBD of NRs has also been shown to feature another hydrophobic exposed groove, also conserved across NR subclasses and susceptible of pharmaceutical attack, called binding function 3 (BF-3). The BF-3 pocket was unexpectedly discovered by X-ray crystallography in the AR and then confirmed through transcriptional assays and site-directed mutagenesis (Estébanez-Perpiñá et al. 2007a). This

concave shaped pocket is located adjacent to the AF-2 groove and comparable in size and depth; furthermore some residues are common to both pockets. BF-3 is composed of H1, H3, the loop between H3 and H4 and H9 and unlike the AF-2 pocket, it does not possess any opposite charge cluster delimiting residues. Mutations in the BF-3 site have been shown in AR to greatly boost its activity suggesting that AR-BF-3 site may be a co-repressor site, although this is still to be verified for AR. Although BF-3 may be regulated by protein binders, its most striking characteristic is that of its interconnection with the AF-2 surface conformation and its role in modulating AF-2 capabilities to engage in contacts with coactivator peptides/proteins. Therefore we could say that BF-3 has been shown to modulate coactivator recruitment via allosteric communication with AF-2 (Estébanez-Perpiñá et al. 2007b; Grosdidier et al. 2012).

3 AF-2 Pocket—Possible Drug Target?

Over the years the disruption of NR-cofactor interactions through targeting the AF-2 function has become a more substantiate target area for pharmaceutical intervention. X-ray crystallographic studies revealed that despite the high sequence homology of NRs AF-2 function, NRs can present different electrostatic characteristics and surface shapes that may be exploited to achieve selectivity. Experiments proved that although most coactivators bind thanks to the LxxLL motif, selectivity of peptide sequences between NRs is dependent significantly on the residues flanking the core motif (Vaz et al. 2009; Teichert et al. 2009; Geistlinger and Guy 2003). Different classes of LxxLL interacting motifs from distinct NRs were elucidated thanks to the studies using large focused combinatorial phage libraries. Peroxisome proliferator-activated receptor (PPAR)-LBD, for example, showed preference for HPLxxLL as a small consensus motif, which aided in the discovery of a peptide (NBM131: sequence ATTPPTLHPLLTQFLRTD) (Mettu et al. 2007). This small peptide was found to have a high affinity for both PPARα (NR1C1) and PPARγ (NR1C2) and to compete with coactivators such as PPARγ-coactivator 1α (PGC1α) and repress PPAR mediated activity, opening a new window into PPAR inhibition. Additional studies with other NRs such as the mineralocorticoid receptor (MR/NR3C2) showed that the small motif MPLxxLL has a high affinity for this receptor and that not surprisingly 50% of all peptides that have been shown to bind to MR to date possess this sequence in their protein sequence. ER-LBD peptide screens with LxxLL core flanked with 7 random amino acids revealed 3 distinct classes of LxxLL interacting proteins (class I: "X_5SRLxxLLX$_7$" class II: "$X_4{}^{H}/_{X}$PLLxxLLX$_7$ and class III: "$X_5{}^{S}/_{T}{}^{L}/_{I}$LxxLLX$_7$") that were able to mimic the interaction between ER and endogenous coactivators and block ER transcriptional activity. From these experiments the peptide #293 (SSIKDFPNLISLLSR) was shown to mimic the interaction between ER and physiological coactivators and block ER transcriptional activity exerting a much higher affinity towards the ERβ isoform rather than ERα. (Patent no. WO1999054728A2) (Chang et al. 1999). Recent experiments

effectively screened the ER surface for novel peptide binders and found a series of proline peptide sequences with the highly evolved PXLxxLLXXP sequence. This sequence consensus reminiscent of the class II peptides described above, was then biochemically and structurally analyzed. The results obtained proved the use of these flanking prolines residues by the peptide for helix binding optimization to the surface charge clamp by determining the precise helix length. Furthermore, the proline residues seem to increase the hydrophobicity of the charge clamp residues, which strengthens the electrostatic interactions and favors more stabilizing hydrogen bond interactions (Fuchs et al. 2013). A new approach towards NR peptide based inhibition was described by Phillips *et al*, whereby ER LxxLL based peptides with improved α-helix stability were synthesized by adding a hydrocarbon link, known as stapled peptides (Phillips et al. 2011).

The AR exhibits a peculiarity that is not shared with other NRs. An important feature of the AR LBD is that its AF-2 pocket interacts with its N-terminal domain (NTD), an inter-domain interaction that has key impact in AR physiological actions. AR-NTD contains two NR-like boxes as the ones found in NR coactivators. Surprisingly these motifs contain an FxxLF sequence (residues 23–27 in the AR-NTD, where F stands for phenylalanine) and WxxLF (residues 433–437 in the AR-NTD, W, tryptophan) sequence and they mediate directly the interaction with the AF-2 groove (He et al. 2000, 2002; Langley et al. 1995; Wilson 2011; Brodie and McEwan 2005). Both motifs ($^{F}/_{W}$xxLF and LxxLL) interact in a similar fashion utilizing the charge clamp amino acid K720 for the stabilization and positioning of the α-helix (Vaz et al. 2009). However, a unique feature of AR is its higher affinity for FxxLF motif containing coregulators. This is most likely due to additional H-bonding interactions of this motif with E897 and the malleability of some of the long side chain residues in this binding site that can accommodate bigger residues such as phenylalanine and allow for extra H-bonding interactions (e.g. I737). This is additionally confirmed by the 1 Å shift difference observed between the two classes of regulatory peptides and could be used to our advantage when designing antiandrogen molecules.

Nevertheless, even though all this information obtained by phage display or physiologically-derived peptides have aided in our understanding of the principles of selectivity of these NRs towards their coregulators and *ergo* their recognition principles, small peptides have limitation in their application in clinical research. It is incredibly challenging to achieve efficient intracellular delivery *in vivo* of these molecules mostly due to their poor stability and permeability as well as their short plasma half-life.

3.1 Small Molecule Based Inhibitors for AF-2: Can They Be Used in the Clinic?

The earliest example of small molecules known to alter the actions of NRs and that bind outside the LBD were described for ERα. These where pyrimidines that

block the interaction of purified estradiol (E2)-activated ERα with labeled SRC1 box 2 peptide in fluorescence polarization assays (Rodriguez et al. 2004) (Table 1). Posterior optimization of these inhibitors led to a pyramidine based inhibitor with an inhibition constant (K_i) value of 2–3 μM using time resolved fluorescence resonance energy transfer (FRET). From then on, many examples of different inhibitors have shown to have effects *in vitro* and *in vivo* against ER (Becerril and Hamilton 2007; Zhou et al. 2007). Examples are amphiphatic benzene inhibitors that proved to inhibit mediated transcription of ERα in the endometrial adenocarcinoma HEC-1 cells (Gunther et al. 2009a), or the guanylhydrazone ERI-05 discovered by high throughput screening and that inhibits endogenous expression of the ERα regulated gene pS2 in the breast cancer MCF-7 cell line at a 20 μM concentration (LaFrate et al. 2008; LaFrate et al. 2009) (Table 1). The first crystal structure of ER co-crystallized with one of these coactivator binding site inhibitors was that of 4-hydroxytamoxifen (4-OHT) (Fig. 2) (Kojetin et al. 2008; Wang et al. 2006a). Tamoxifen, which has been used for more than 30 years, is one of the most effective treatments for ER-positive breast cancer. This inhibitor, which has been shown to bind to the LBP, had previously hinted that its total binding capacity to ERα was 2 times that of estradiol, the crystal structure ERβ-4-OHT surprised the scientific community by demonstrating that 4-OHT binds both to the AF-2 groove and the expected LBP (Kojetin et al. 2008; Jensen and Khan 2004). The binding of this 4-OHT to the surface appears to be mostly hydrophobic with numerous van der Waals interactions helping to accommodate this inhibitor perfectly in the hydrophobic patches of the

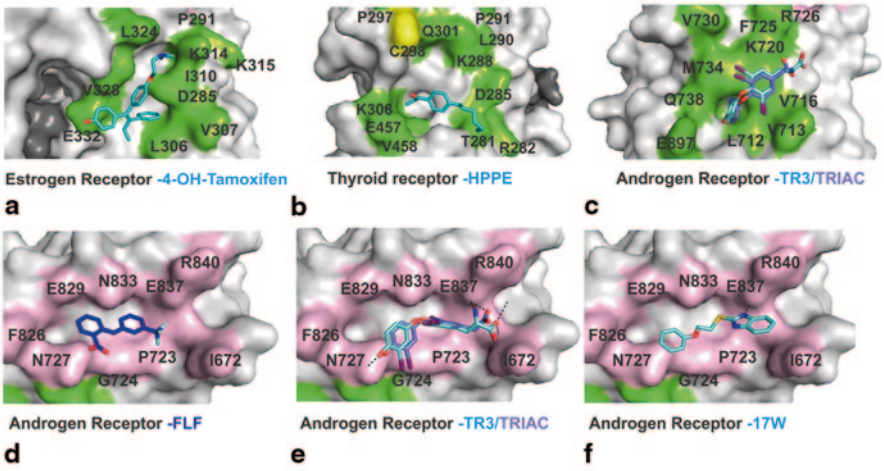


Fig. 2 Surface representations of AF-2 and BF-3 binding sites: residues involved in AF-2 are shown in green while residues involved in BF-3 are depicted in pink. Inhibitors bound to these sites are shown in stick form; carbons are depicted in blue while other element follows the CPK coloring system. **a** AF-2 binding site of ER co-crystallized with 4-OHT. **b** AF-2 binding site of TR co-crystallized with HPPE. **c** AF-2 binding site of AR co-crystallized with inhibitors T3 and TRIAC; AR BF-3 binding site co crystallized with. **d** FLF **e** T3 and TRIAC and **f** compound 32 (2-((2-Phenoxyethyl)thio)-1 H-benzimidazole)

Table 1 List of inhibitors that have been proven to bind to NRs alternative sites. The list includes the chemical structure of each inhibitor, in which pocket they bind to the NR, if there is a crystal structure and if so the Protein Data Bank (PDB) number as well as the activity these inhibitors have shown and where they have been published

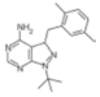
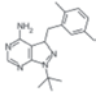
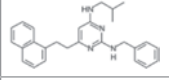
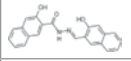
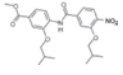
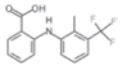
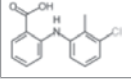
NR	Molecule	Chemical structure	Binding site	Co-structure (POB)	Activity	Publication
ER (NR3A)	Pyrimidines		AF-2	No	Blocks interaction of purified E2 activated ERα with SCR1-Box 2	Rodriguez et al. 2004
ER (NR3A)	ERI-05		AF-2	No	Inhibits endogenous expression of pS2 (ERα regulated protein) in MCF-7 cells	Lafrate et al. 2008
ER (NR3A)	4-OH-Tamoxifen		Af-2/LBP	Yes (2FSZ)	Major tamoxifen metabolite, acts as a mixed agonist/antagonists. Used as an anti-estrogen therapy drug in hormone receptor positive breast cancer in pre-menopausal women	Wang et al. 2006a
ER (NR3A)	Bicyclo[2.2.2]octane		AF-Z	No	Partial inhibition of the coactivator recruitment to ERα as shown by TR-FRET assay	Zhou et al. 2007
ER (NR3A)	2(Amphipathic Benzene)		AF-2	No	Inhibit ERα mediated transcription in HEC-1 cells	Gunther et al. 2009a
TR (NR1A)	GC-24		dimer interface/ LBP	Yes (1Q4X)	TR-β selective compound that mimics physiological action of thyroid hormone	Borngraeber et al. 2003
TR (NR1A)	DHPPA		AF-2	Yes deaminated-product HPPE (2PIN)	Prevents binding of SRC-2 peptide in fluorescence polarisation (FP). High cytotoxicity in both TR-positive/negative cancer cell lines	(Arnold et al. 2007; Estébanez-Perpiñá et al. 2007c)
TR (NR1A)	2{31,2}		AF-2	No	Prevents binidng of SRC-2 peptide in fluorescence polarisation (FP), Significantly inhibits T3-mediated luciferase activity	Hwang et al. 2011

Table 1 (continued)

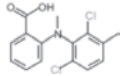
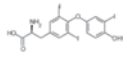
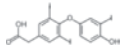
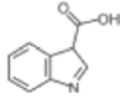
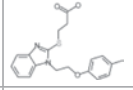
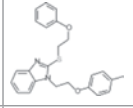
NR	Molecule	Chemical structure	Binding site	Co-structure (POB)	Activity	Publication
TR (NR1A)	T3		AF-2/other sites	Yes (4LNW)	T3, natural ligand from TR	Souza et al. 2014
VDR (NR1\1)	Benzodiazepine 2		AF-2	No	Showed binding to VDR in a TR-FRET assay. VDR mediated tracription in presence of increased concentration of natural ligand showed no dose-response curve	Mita et al. 2010
VDR (NR1\1)	3-indolyl- methanamine 31b		AF-2	No	FP-SRC-2 based assays proved to disrupt VDR-SRC-2 interaction. Inhibits VDR mediated transcriptionas at cellular level	Nandhikonda et al. 2012
PXR (NRl\2)	Ketoconazole		AF-2	No	Interaction of activated PXR with SRC-1 was achieved in a non competitive fashion. Scitillation proximity assays using PXR. LBP ligand [3H]SR12813 and site-directed mutagenesis	Wang et al. 2007
PXR (NRl\2)	Leflunomide		AF-2	No	Disrupts PXR/SRC-1 interaction by binding to AF-2 of PXR as shown by site directed mutagenesis and molecular docking studies	Ekins et al. 2007
PXR (NRlI2)	Coumestrol		AF-2/LBP	No	Inhibits SRC-1 recruitment by PXR. FP competition experiments with fluorescently labeled LxxLL of SRC-1 suggest a second binding to AF-2 (originally bound to LBP)	Wang et al. 2008
Nur77 (NR4A1)	TMY		other sites	Yes (3V3Q)	Releasesoff LKB1 to the cytoplasm to phosphorylate AMPKα. Reduces blood glucose and alleviates insulin resistance in type II diabetes and high-fat diet- and streptozotocin- induced diabetic mice	Zhan et al. 2012

Table 1 (continued)

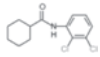
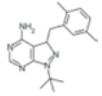
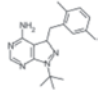
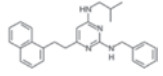
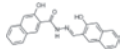
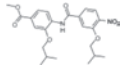
NR	Molecule	Chemical structure	Binding site	Co-structure (POB)	Activity	Publication
Nur77 (NR4A1)	THNP		other sites	Yes (4JGV)	Induces melanoma cell death and bearly causes cell death in anyother type of cancer cell line. Inhibits the formation of tumors in the liver and lung in a Nur77 dependent manner. Nix-Nur77 interaction	Wang et al. 2014
Nur77 (NR4A1)	DPDO		other sites	Yes (4KZI)	Cannot facilitate Nix and Nur77 binding. Does not have any effect on cells or in vivo	Wang et al. 2014
AR (NR3C4)	Kl0		AF-2	Yes (2PIP)	Inhibition of AR and SRC2-3 binding detected by FP screens.	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	RB-1		AF-2	Yes (2PIQ)	Inhibition of AR and SRC2·3 binding detected by FP screens	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	2-metylinciole		AF-2	Yes (2PIO)	Inhibition of AR and SRC2-3 binding detected by FP screens	Estébanez-Perpiñá et al. 2007a
AR (NR3G4)	Pyrimidines		AF-2	No	Achieved AR over ER selectivity as determined by AR transcriptional activated cellular assay. Poor solubility	Gunther et al. 2009b
AR (NR3G4)	Diarylhydrazides		AF-2	No	Induce prostate specific antigen expression in absence of androgens. Inhibit DHT and cyproterone acetate stimulated prostate specific antigen	Axerio-Cilies et al. 2011
AR (NR3C4)	D2		AF-2	No	Blocks androgen-induced nuclear uptake of genomic activity of AR. Abrogates androgen- induced proliferation in PCA cells in vitro. Inhibits tumour growth in a mouse xenograft model	Ravindranathan et al. 2013

Table 1 (continued)

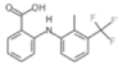
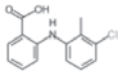
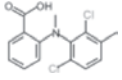
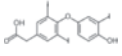
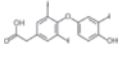
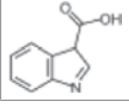
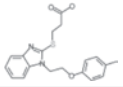
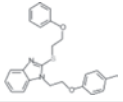
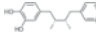
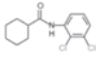
NR	Molecule	Chemical structure	Binding site	Co-structure (POB)	Activity	Publication
AR (NR3C4)	Flufenamic acid		AF-2/BF-3	Yes (2PIX)	AR-LBD interaction with radiolabeled SRC-2 in vitro was inhibited. AR activity was inhibited in culture cells. AR activity was inhibited in culture cells. DHT response with AR-LBD tethered to a reporter with GAL-DNA binding function and full length AR proved a dose dependent inhibition	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	Tolfenamic acid		AF-2/BF-3	No	AR activity was inhibited in culture cells. DHT response with AR·LBD tethered to a reporter with GAL-DNA binding function and full-length AR proved a dose dependent inhibition	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	Mectofenamic acid		AF-2/BF3	No	AR activity was inhibited in culture cells. DHT response with AR·LBD tethered to a reporter with GAL·DNA binding function and full-length AR proved a dose dependent inhibition	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	T3		AF-2/BF-3	Yes (2PIW)	T3-inhibits androgen-induced gene expression. However is a partial agonist of T877-AR mutatnt	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	TRIAC		AF-2/BF-3	Yes (2QPY)	Protein precipitates in pull down assays. AR activity was inhibited in culture cells. DHT response with AR-LBD tethered to a reporter with GAL-DNA binding function and full-length AR proved a dose dependent inhibition	Estébanez-Perpiñá et al. 2007a
AR (NR3C4)	Indole-3-carboxylic acid		BF-3	Yes (2PIP)	Inhibition of AR and SRC2-3 binding detected by FP screens.	Estébanez-Perpiñá et al. 2007a

Table 1 (continued)

NR	Molecule	Chemical structure	Binding site	Co-structure (POB)	Activity	Publication
AR (NR3C4)	ZINC03877300 (compoond 2)		BF-3	Yes (2YLQ)	Inhibits AR transcriptional activity in cell based eGFP screening assays	Lack et al. 2011
AR (NR3C4)	ZINC02058890 (compoond 3)		BF-3	Yes (2YlO)	Inhibits AR transcriptional activity in cell based eGFP screening assays	Lack et al. 2011
AR (NR3C4)	ZINCOOO12342(compoond 4)		BF-3	Yes (3ZQT)	Inhibits AR transcriptional activity in cell based eGFP screening assays	Lack et al. 2011
AR (NR3C4)	MJC013		BF-3	No	Inhibits AR function by preventing hormone-dependent dissociation of Hsp90-FKBP52-AR complex. This inhibitor also inhibits AR-dependent gene expression and androgen stimulated prostate cancer cell proliferation	De Leon et al. 2011
AR (NR3C4)	2- ((2- Phenoxyethyl) thio)-lH-benzimidazole		BF-3	Yes (4HLW)	Demonstrates significant antiandrogen potency against LNCaP and enzalutamide-resistant PCa cell lines	Munuganti et al. 2013

coactivator-binding groove (Kojetin et al. 2008; Wang et al. 2006a; Wang 2006b). The binding of 4-OHT to the ER AF-2 pocket may account for the mixed agonist/antagonist activity of type I antiestrogens; nevertheless this discovery opens potential therapeutic avenues for improved antiestrogens.

As for ER, the function of most NRs has been modulated through the modulator binding to LBP. This was the case for TR where alterations in function was achieved mostly through analogues of the thyroid hormone (T3), for instance in the treatment of hypothyrodism. The first description of a non-LBP binding inhibitor for TR was that of a β-amino ketone. Arnold *et al*. carried out a high through put screen (HTPS) utilizing SRC2 box 2 peptide as a fluorescent probe for fluorescence polarization (FP) assays with the TRβ isoform. This HTPS led to a group of α/β unsaturated ketones that were shown to irreversibly bind to an exposed cysteine on the surface of the AF-2 groove of TR, with the best 3-(dibutylamino)-1-(4-hexylphenyl)-propan-1-one (DHPP) having an IC_{50} of 2 μM (Table 1). Several lines of evidence including TRβ mutants and mass spectroscopy (MS) analysis have led to direct evidence of a covalent adduct formation of these inhibitors to Cys298 in TRβ. This reaction has been shown to occur in two steps, initial beta elimination of the molecule DHPPA to form 1-(4-hexylphenyl)-prop-2-en-1-one (HPPE) followed by a covalent bond formation between HPEE and TRβ. This is also consistent with the fact that a deamination reaction of DHPPA would not be feasible at a physiological pH without the aid of another active molecule. For DHPPA this mechanism is catalyzed directly on the protein surface. X-ray crystallographic studies of DHPPA with TRβ showed the deaminated active principle in the AF-2 groove supporting the specific mechanism of alkylation and deamination *in situ* catalyzed by Cys298 (Fig. 2b). These compounds were extensively studied and optimized using a structure-activity-relationship (SAR) approach. Though potency measured through FP did not seem to improve other important qualities such as solubility, permeability, cytotoxicity and selectivity towards TR over AR, ERα and PPAR were greatly improved (Arnold et al. 2007; Estébanez-Perpiñá et al. 2007c, d; Arnold et al. 2006). Other HTPS have led to the discovery of methylsulfonylnitrobenzoates (MSNB) inhibitors (Hwang et al. 2011). MS and mutational studies demonstrated that these inhibitors also bind to TR irreversibly by targeting the Cys298 residue in the AF-2 pocket. Selectivity towards TRβ over AR and PPARγ as well as good solubility and cytotoxicity profiles made these inhibitors highly interesting. The most potent MSNB inhibitor was MI151 with a potency of 1.8 μM in the FP-SRC2 interaction assay with TRβ showed less off target activity than DPPAH (Hwang et al. 2011). These MSNB inhibitors were further optimized by the removal of the ester linker between the methylsulfonylnitro and the rest of the molecule to a cyclic bioisostere, such a thiazole. These inhibitors were therefore termed sulfonylnitrophenylthiazoles (SNPTs) and showed improved *in vivo* properties as well as the classical irreversible activity through Cys298 binding. Two inhibitors showed low μM potency, that of 2{4,1,5} and 2{3,1,2} showed by FP-SRC-2 binding assays to have an IC_{50} of 0.3 and 2.4 μM respectively however the less active of these two presented additionally good transcriptional inhibitory activity (5 μM in the T3-mediated luciferase assay) (Hwang et al. 2012) (Table 1).

Inhibition of NRs through their AF-2 binding pocket has only been minimally attempted in other NRs. Two AF-2 small molecule inhibitors have been described for the vitamin D receptor (VDR/NR1I1) and although there is no *in vivo* data of their inhibition, they have proven to bind to this NR *in vitro* by a TR-FRET assay. The first non-LBP binding inhibitors for the VDR were the benzodiazepines derivatives (Mita et al. 2010) (Table 1). These inhibitors proved to affect the interaction between VDR and LxxLL coactivator peptides, whereby the most potent of the group showed an IC_{50} of 20 μM in a VDR TR-FRET based assay (Mita et al. 2010). Monitoring of the effect of benzodiazepines in VDR mediated transcription, in the presence of increased concentration of its natural ligand (1,25 dihydrozyvitamin D3), showed no shift in the dose response curve, confirming the non-LBP binding nature (Mita et al. 2010). It was then shown that this inhibitor acts as an α-helix peptidomimetic inhibitor, whereby the three alkyl side chains presented in this inhibitor mimic the three leucines in the LxxLL motif. The tetrahydrobenxodiazepinamine therefore acts as a rigid structure that mimics the α-helical arrangement and the methylbutene and the two isopentane groups mimic the leucines in the "NR box" motif of coactivators (Mita 2010). In a separate study, small molecule 3-indolylmethanamides were identified as alternative site binders for the VDR LBD alternative binding was that of (Nandhikonda et al. 2012) (Table 1). These molecules block the interaction of VDR with SRC-2 in FP-based assays as well as inhibit VDR mediated transcriptions at a cellular level and presented with good selectivity towards VDR over AR, ERβ, TRα, TRβ and PPARγ (Nandhikonda et al. 2012). Surprisingly this indol-methanamine proved to bind to VDR irreversibly involving a reaction mechanism, whereby an azafulvenium salt is produced as an intermediate compound forming a positive like species, which can act with any nucleophile in the surface of VDR (Nandhikonda et al. 2012). However this promising inhibitor which has an IC_{50} in the low micromolar range proved to have high cytotoxicity that is yet to be determined if it is due to the irreversible mechanism observed (Nandhikonda et al. 2012). More *in vivo* data is required in order to see if this inhibitor is a viable option for clinical inhibition of VDR.

Another example of non-LBP bound inhibitors was shown for the NR pregnane X receptor (PXR/NR1I2). PXR, as well as the constitutive androstate receptor (CAR/NR1I3), are two orphan NRs, meaning that no endogenous ligand has been identified as of yet (Willson and Kliewer 2002). However, it has been shown that they can function as xenobiotic sensors as they interact with many foreign chemicals (Biswas et al. 2009). Antagonists for PXR are of great interest due to their impact on the therapeutic efficiency of drugs and their ability to prevent harmful drug-drug interactions. Nevertheless, this is a highly complicated task as their LBP is highly flexible and can accommodate many ligands as has been repeatedly shown in the many structures of PXR co-crystallized with different ligands of different sizes (Xue et al. 2007; Watkins et al. 2001; Chrencik et al. 2005; Cheng and Redinbo 2011). The first non-LBP ligand described for PXR is ketoconazole, a known antifungal drug that is also used as an antiandrogen for metastatic PCa (Peer et al. 2014; Huang et al. 2007) (Table 1). This molecule antagonizes PXR in the presence of the antibiotic rifampicin, a known agonist of PXR and was also shown to disrupt

the interaction of activated PXR with SRC-1 in a non-competitive fashion (Huang et al. 2007). Furthermore, the non-LBP binding nature of ketoconazole to PXR was confirmed through scintillation proximity assays, which excluded a potential LBP competitive mechanism. This was further confirmed by site-directed mutagenesis. Other inhibitors have been shown to bind to AF-2 such as the phytochemical coumestrol, found to bind to the surface of PXR in functional gene reporter assays and to antagonize PXR ligand dependent SRC-1 recruitment to the same extent regardless of the concentration of rifampicin (Want et al. 2008). FP-competition experiments with fluorescently labeled SRC-1 LxxLL ligands confirmed its binding to AF-2 (Huang et al. 2007; Wang et al. 2007; Ekins et al. 2007). This inhibitor was found to be highly selective for PXR except for some cross-reactivity with ER. The drug leflunomide is used for rheumathoid arthritis and is another inhibitor which was found to show inhibition of the PXR/SRC-1interaction and proved by site-directed mutagenesis, to directly bind to AF-2 (Ekins et al. 2008). However, more structural studies are needed to view the binding positioning of these inhibitors and additional *in vivo* data of these inhibitors has not been obtained to date (Ekins et al. 2007, 2008).

ER, TR, PXR and VDR are all NRs whose AF-2 function has been shown to be a viable druggable non-LBP target. Nonetheless following ER, AR is the second NR whereby its non-LBP binding pockets have been extensively exploited for rational drug design. The first non-LBP binding inhibitors for AR were based on the pyrimidines inhibitors, initially designed for ER (Gunther et al. 2009b). The authors rationally designed tri-substituted peptidomimetic pyrimidines with bulkier groups than those initially utilized for ER emulating the AR preference for bulkier side chains (i.e FxxLF) in the NR boxes of co-regulatory proteins (Gunther et al. 2009b). This classical peptidomimetic approach achieved pyrimidines that were AR selective over ER and that had IC_{50} values ranging 1.5–6.6 μM as determined by AR inhibition in a transcriptional activated cellular assay (Gunther et al. 2009b).

Axerio-Cilies *et al.* carried out a structure-based drug design approach using all the structural information available on AR and its binding to different coactivators (Axerio-Cilies et al. 2011). This study led to a set of molecules with a rigid linker and two aromatic features that mimicked an FxxLF NR box: the diarylhydrazide inhibitors (Axerio-Cilies et al. 2011; Caboni et al. 2012). With high selectivity for AR over GR (NR3C1), ERα/β, but still showing some inhibition of PR, these inhibitors were shown to inhibit AR coactivator binding around 13–26 μM IC_{50} in a TR-FRET assay. Furthermore, they induce prostate specific antigen (PSA) expression in the absence of androgens and to inhibit DHT and cyproterone acetate stimulated PSA (Axerio-Cilies et al. 2011; Caboni et al. 2012) (Table 1). A further example of the utility of peptidomimetics for AR targeting was published recently by Ravindranathan et al. (2013). This group evaluated the X-ray structure of peptide coactivator sequence LxxLL/FxxLF bound to AR and designed two compounds mimicking these coactivator motifs. The main structural scaffold was that of a bis-benzamide group where two benzyl groups of compound D1 mimicked FxxLF motif and in the analogous compound D2, two isobutyl groups were used to mimick the LxxLL motif (Ravindranathan et al. 2013). Surprisingly, D1 did not show any inhibitory ef-

fect in AR-coactivator binding, however, D2 was shown to block the interaction of many coregulator proteins in co-immunoprecipitation assays using the LNCaP cell line (Ravindranathan et al. 2013). D2 also proved to block AR dependent transcriptional activity and prevent AR nuclear translocation as well as inhibiting AR-mediated PCa cell proliferation. Furthermore, it was reported to inhibit AR-dependent growth of xenograft tumors *in vivo* and AR expression in human tumors cultured *ex vivo* (Ravindranathan et al. 2013). These findings provide evidence that targeting the AF-2 binding site of AR using peptidomimetics may be a viable therapeutic approach for patients with advanced PCa.

Other functional FP screens with the aim of identifying potential PCa treatment molecules binding to the AF-2 function identified the off-patent nonsteroidal anti-inflamatory drugs (NSAIDs) and thyroid hormones as potential antagonists of AR (Estébanez-Perpiñá et al. 2007a; Buzón et al. 2012). This screen led to the identification of: (i) two kinase inhibitors, 1-*tert*-butyl-3-(2,5-dimethyl-benzyl-)-1 H-pyrazolo[3,4,-D]pyrimidin-4-ylamine (K10) and 3-((1-tert-butyl-4-amino-1 H-pyrazolo[3,4D]pyrimidin-3-yl)methyl)phenol (RB-1); (ii) five small molecules, flufenamic acid (FLF), tolfenamic acid (TOL), meclofenamic acid (MEL), 2-methylindole and indole-3-carboxylic acid; and (iii) two thyroid hormones, triiodothyronine (T3) and 3,5,3'-triiodothyroacetic acid (TRIAC) (Table 1). The complex structure of the two kinase inhibitors with AR proved their binding preference to the AF-2 binding site. These inhibitors form a plethora of hydrophobic interactions and are additionally stabilized by H-bonding to the residue M734. RB-1 was shown to form an additional H-bond with the residue K720, a key residue that forms part of the "charge clamp" residues that normally stabilize the LxxLL motifs in their binding to the AF-2 site. Some small molecules such as 2-methylindole also showed by crystallographic data to be located in the AF-2 occupying the same subpocket of the AF-2 groove as the pyrimidine moiety of RB-1 and K10. The two thyroid hormones T3 and TRIAC also proved to bind to this coregulator binding groove, however these two inhibitors seem to not form any hydrogen bonds and be stabilized uniquely by week hydrophobic interactions (Fig. 2c). These two thyroid hormone compounds and some other small molecules in this screen proved to additionally bind to the exposed BF-3 pocket (Fig. 2e).

4 A Neighboring Pocket Emerging as a Possible Drug Target Site–The BF-3

Additionally to those AF-2 binding molecules identified in the FP-screen by Estébanez-Perpiñá *et al.* and contrary to what was expected, the X-ray crystallographic data did not reveal the TRIAC or T3 uniquely in the AF-2 groove but showed an additional strong uniformly well defined electron density in another novel surface pocket named BF-3 (Estébanez-Perpiñá et al. 2007a). Furthermore, the NSAID called flufenamic acid (FLF) was shown to be uniquely bound to the BF-3 pocket of AR (Fig. 2d). Moreover, the TRIAC and T3 inhibitor proved to interact pref-

erentially at the BF-3 binding site versus in Cursive the AF-2. In the BF-3 these inhibitors form many hydrophobic interactions and H-bonds with the residues N727 and E837, strongly securing the inhibitors in place (Fig. 2e). In the case of T3 an additional hydrogen bond is observed between the carboxyl group of T3 and the protein backbone and side chain of residue I672. On the other hand, the small molecule FLF showed uniquely hydrophobic interactions with the BF-3 surface (Fig. 2d) (Estébanez-Perpiñá et al. 2007a). Since the discovery of the BF-3 pocket many other AR inhibitors have been identified to bind to this non-LBP binding site. De Leon *et al.* elucidated a lead compound, termed MJC013, a dichloropheyl-cyclohexanecarboxamide molecule, which inhibits AR function by preventing hormone dependent dissociation of the Hsp90-FKBP52-AR complex and that uniquely binds to the BF-3 binding site in AR, as shown by functional studies (De Leon et al. 2011).

Furthermore, recently a large virtual screen, performed by Lack *et al.* using the Zinc database and AR LBD crystal structure, lead to the identification of several different scaffolds of inhibitors that proved to inhibit AR both in eGFP screening assays and in AR transcriptional luciferase assays using LNCaP and HeLa cells (Irwin et al. 2012; Irwin and Shoichet 2005; Lack et al. 2011). The highly selective compounds 1–4 (ZINC03445992/ZINC03877300/ZINC02058890 and ZINC00012342 respectively) were analyzed by means of X-ray crystallography, which proved that all these four inhibitors bound to BF-3 except compound 1 which was shown to be bound to BF-3 and AF-2 (Lack et al. 2011) (Table 1). Compounds 1 and 3 were shown to be stabilized mainly by hydrophobic interactions; meanwhile compounds 2 and 4 formed additionally hydrogen bonding interactions with residue N727, a key residue of both the AF-2 and BF-3 pockets. This work proved the possibility of successfully screening for inhibitors incapable of repressing AR transcriptional activity and binding to the BF-3 pocket, going from 10 million commercially available compounds to 8 possible candidates. However further *in vivo* data of these inhibitors is required. From this extensive report the same group carried out a further optimization of a set of compounds elucidated, with a core thiol-benzimidazole structure (Lack et al. 2011; Munuganti et al. 2013). Based on the previous crystal data obtained, the authors decided to remove the 2-(4-methylphenoxy)ethanamine group, a moiety of the compound which did not seem to form any essential interactions for stabilization in the BF-3 pocket. This led to a functional core for rational drug design, the 2-[(2-Phenoxyethyl)thio]−1 *H*-benzimidazole (compound 32), which inhibited AR transcriptional activity 3-fold stronger (IC_{50} 4.2 μM) (Munuganti et al. 2013). The crystal structure showed a well defined electron density and positioned the inhibitor in the BF-3 binding site where it makes van-der-Waals interactions as well as a strong hydrogen bond with E837 (Munuganti et al. 2013). Various SAR studies were performed with compound 32, in order to obtain improved derivatives; replacement of the oxygen atom in SC2H4O did not improve the potency of the inhibitor, however addition of small hydrophobic substituents in the benzene ring was able to enhance anti-AR potency leading to two compounds in the low μM range (compound 47 and 49 containing methyl at meta and diortho positions respectively). Furthermore, these compounds did not only prove to be specific for AR, compared

to ERα, but to additionally exhibit effective growth inhibition in both LNCaP and ENZ-resistant cell lines (Munuganti et al. 2013). All these inhibitors confirm that this BF-3 orientated compounds show a different anti-AR action to clinically used anti-androgens and with little cell toxicity, however *in vivo* studies are still required in order to fully understand the physiological effect of inhibiting BF-3 pocket.

All of these BF-3 targeting inhibitors here presented have been designed to alter AR function; however the in-depth analysis of these structures as well as other NRs X-ray structures proved that this BF-3 pocket is conserved amongst different NRs (Estébanez-Perpiñá et al. 2007a; Buzón et al. 2012). Opening a new targeting site for non-LBP inhibitors, nevertheless little is known on the structural/functional relationship of NRs binding to coregulators, mutational studies in the BF-3 site hint at the possibility of the BF-3 surface allosterically communicating with the AF-2 groove influencing coactivator recruitment as a consequence (Estébanez-Perpiñá et al. 2007a).

5 Communication and Allosteric Mechanisms Between AF-2 and BF-3

As previously mentioned AF-2 can undergo subtle induced fit rearrangements upon coactivator binding and several residue side chains move to create a larger hydrophobic pocket that will accommodate the bulky hydrophobic side chains, like in the case of the AR that can accommodate W/FxxLF motifs present in the AR-NTD and AR specific coactivator such as ARA70. Several of the compounds mentioned above, including TRIAC and members of the fenamic acid series of anti-inflammatory compounds, although having been shown to preferentially bind to BF-3, still inhibit AR/LxxLL, which occurs in the AF-2 groove (Grosdidier et al. 2012). In the case of AR, crystal structures with and without TRIAC suggests that compounds binding to BF-3 trigger allosteric alterations that propagate to AF-2 and inhibit coregulator binding (Estébanez-Perpiñá et al. 2005b; Grosdidier et al. 2012). All these results suggest allosteric communication between the two pockets and have been complemented by many mutational studies (Estébanez-Perpiñá et al. 2005b; Grosdidier et al. 2012; Buzón et al. 2012). The biggest characterization of this AF-2/BF-3 allosteric communication has been undertaken for the AR. Mutations of residues in the interface of these two pockets (Fig. 1), such as N727K, have been shown to make the AR behave as a ‘super AR’ variant. Surprisingly, mutants such as N833R, which is 6.8 Å away from NR727K, and R840A, which is 22.0 Å away from NR727K, show the same results. Additionally, other mutations such as R840E totally abolish AR LBD activity *in vitro* while albeit maintaining its 3D structure or only moderately enhance (F826L/N727K) or inhibit (F826R/R726L) activity (Estébanez-Perpiñá et al. 2007a; Grosdidier et al. 2012). These mutations in BF-3, distant from AF-2 surface, have additionally been shown to have an effect on AR-LBD activation by GRIP1 and N/C interdomain interaction, however with

a completely different pattern, which further supports the effect of these mutations in the structural rearrangement of AR (Askew et al. 2011). This connecting network is of great importance due to the effect that mutations in BF-3 have in coregulator binding that has been observed to lead different diseases, such as that of PCa (E670, Ile672, L830) and androgen insensitivity syndromes (AIS) (L830, P723, E724, R840) in AR (Estébanez-Perpiñá et al. 2007d). However, further studies must be undertaken in order to fully understand this intra-domain mapping network that NRs have repeatedly demonstrated to have, proving its plasticity and environmental adaptability.

6 LBD Dimerization Interface—Interesting Approach?

Most NRs have been shown to exert their transcriptional function as a homo- and/or hetero-dimers. This ability to dimerize is essential for their function and usually occurs initially in the LBD through the H9 of one monomer and H10/H11 of the other. However, different NRs differ slightly from each other's dimerisation mechanism, allowing for a specific/individual targeting of these proteins using this alternative area of the LBD. For example in the NR ERR, which has three different isoforms ERRα/β/γ (NR3B1/NR3B2/NR3B3), ERRγ homodimerization and enhances transactivation while heterodimerization with ERRα inhibits transcriptional activities of both ERRα and ERRγ (Gearhart et al. 2003; Gerhard 2004).

Although at embryonic stages, dimerisation targeting has been attempted in order to block ER function. Chakraborty *et al.* presented an extensive study of ERα dimerisation characterization *in silico* using the X-ray structure of ERα-LBD bound to the ligand DES (PDB: 3ERD) (Chakraborty et al. 2013). H-bonding contacts between the two dimers occur mainly from the N-terminal portion of H10/H11 (these helices are not separated in ER) and the H9 of the other monomer. Three main regions of contact have been described; region I (DKITD-monomer A with QQQHQRLAQ-monomer B), region II (QQQHQRLAQ-monomer A with DKITD-monomer B) and region III (LSHIRMMSNK-monomer A with LSHIRMMSNK-monomer B). These regions are unaltered by the presence or absence of DES and show a high polar environment with distinct patches of positive/negatively charged regions. YP537 (sequence CNVVPLYPDLLLE) was the first peptide to inhibit ER without binding to AF-2 groove (Arnold and Notides 1995). It was then suggested that this inhibitor binds as a "dimerisation inhibitor". This peptide was shown to bind in the human ER dimerisation site and precipitate independently to hormone bound or unbound ER (apo state as well as when ER was incubated E2, 4-OHT and ICI182,780). However it must be mentioned that protein misfolding and aggregation are not likely to be the best option in a therapeutic context due to functional *in vivo* consequences not being straightforward and that NRs are generally bound to chaperones in their inactive form in order to prevent misfolding. Other *in silico* experiments have identified an extended and a mutant version of the bind-

ing sequence motif region II a foremost mentioned, the LQQQHQQLAQ sequence which has been proposed to inhibit dimerisation in liganded and un-liganded ERα (Chakraborty et al. 2012).

Evidence of the dimerisation interface as a possible drug target was also observed for TRβ. The crystallographic data of the inhibitor, GC-24, with the receptor LBD contained an electron density for GC-24 in the LBP, but also an additional one for a second GC-24 molecule, placed in the potential dimer interface of TR (Fig. 3) (Borngraeber et al. 2003). The additional molecule is clearly seen bound in a surface pocket formed by H1, H9, H10 and H11 in the opposite site of the AF2 (Fig. 3b). This fact is consistent with the fact that the crystallization conditions utilized by the group were different to those previously described by others, as these did not yield any crystals (Borngraeber et al. 2003). A recent paper also reported a new X-ray crystallography structure of TRα-LBD that depicted a second binding site for T3 and T4, TR ligands, located between H9, H10 and H11 forming H-bonding with residues Q342, E339 and R375. These two pockets have also been observed in the NR growth-inducible immediate early gene nur/77-like receptor (Nur77) (NR4A1). A recent screen of a Nur77 targeting compounds led to the discovery of 1-(3,4,5-trihydroxyphenyl)nonan-1-one (THNP) and its derivative 1-(3,5-dimethoxyphenyl)decan-1-one (DPDO) (Souza et al. 2014; Wang 2014). THNP has recently been shown to have an integral role in melanoma autophagic cell death via Nur77 (NR4A1) inhibition. The complex structure of THNP depicted this inhibitors bound between H9, H10 and H11, an area that the authors term Site C (Wang et al. 2014). An overlay of the THNP-Nur77 structure with that of TR-T3 complex proved both these ligands to be bound in the same area. Surprisingly THPN and not DPDO was shown to facilitate the interaction with Nur77 through the binding to this site C with leads to the formation of tumors in the liver and lung in a Nur77 dependent manner (Wang et al. 2014). Comparison of both crystal structures of THPN and DPDO proved that although both these ligands bind between H10-H11 and H9, DPDO interacts at a location close to but not at site C which may explain the inability of DPDO to form the LBD-interacting surface for Nix that results in the lack of DPDO-associated Nur77 targeting to mitochondria and melanoma cell death (Wang et al. 2014).

Remarkably, the binding of another compound ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl]acetate (TMPA) to Nur77 was found in its complex structure to be bound to the same site as previously shown for GC-24 in TR (site between H1, H9, H10 and H11 in the opposite site of the AF2) (Fig. 3b) (Souza et al. 2014; Zhan et al. 2012). This compound was shown to effectively reduces blood glucose and alleviates insulin resistance in type II diabetes high-fat diet– and streptozotocin-induced diabetic mice. Other compounds inhibiting Nurr1 in this same site have been also reported (Zhan et al. 2012; Volakakis et al. 2006).

These results prove that these surface pocket can be considered additional druggable sites and that they can produce desirable *in vivo* results to therapeutic intervention of NRs based diseases (Souza et al. 2014; Zhan et al. 2012).

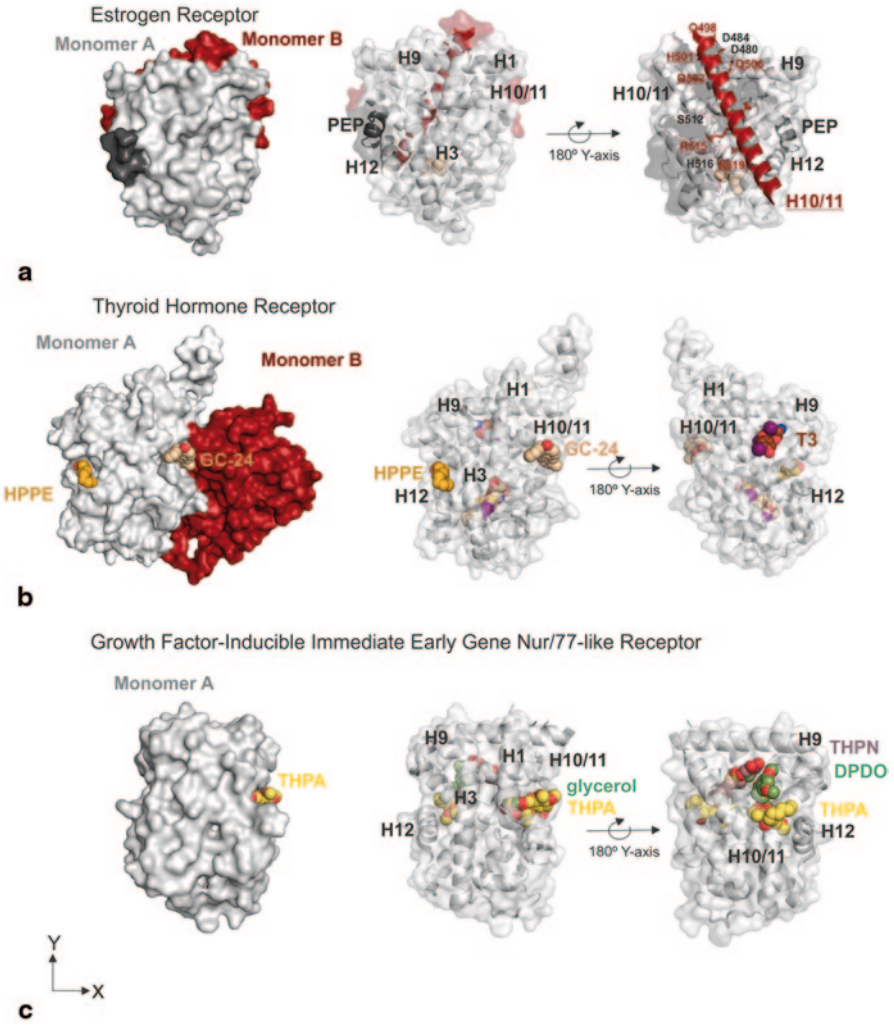


Fig. 3 **a** Surface representation of standard view of ER-LBD followed by monomer. B (depicted in *dark red*) and ER-LBD of monomer A. Cartoon and surface representation in the standard orientation followed by 180° turn on the y-axis showing H-bonding contacts between the two monomers occur mainly from the N-terminal portion of H10/H11 monomer B and the H9 of monomer A as well as between H10/H11 of monomer B and H10/H11 of monomer A, hereby depicted with a *black dotted line*. **b** Surface representation of an overlay of TR crystal structure (PDB: 1Q4X, 4LNW and2PIN) 2PIN structure proved to crystallized as a dimer (monomer A and monomer B are depicted in *grey* and *red*) AF-2 binding inhibitor HPPE shown in *spheres* and *orange* color. Cartoon representation of TR with structure bound to GC-24, HPPE and T3 in an alternative binding site (shown in *sphere* representation and depicted in wheat, *orange* and *brown* color) as well as a turn of 180° on the y-axis showing. **c** Surface representation of Nur77 (PDB:3 V3Q), inhibitor bound to an alternative binding site located between H1, H9, H10 and H11 in the opposite site of the AF2. Followed by a cartoon representation of an overlay of three structures (PDB: 4JGV, 4KZI and 3 V3Q) in standard orientation and a turn of 180° on the y-axis, inhibitors THPN, DPDO and THPA are shown in sphere representation in colors *purple*, *green* and *yellow* respectively

7 Conclusions

The spotlight of NR targeting has moved beyond the LBP due to known alterations of this site in pathological disease. Over the last decade significant evidence has emerged indicating the therapeutic potential of other binding sites, such as AF-2, and small peptides/molecules have demonstrated the possibility of coregulator-NR binding disruption. Techniques such as X-ray crystallography have been essential to understand these binding sites and have led to the discovery of the BF-3 surface-exposed groove. Although targeting this site has been proven in AR further evidence of the therapeutic potential of this site in other NRs is required. However, the conservation of certain key amino acids and the clear allosteric effect between these two sites renders the BF-3 site as an attractive therapeutic option as well. Other approaches, such as dimerisation disruption of NRs, are currently flourishing and although they are still in their infancy, these approaches have proven to be viable possibilities. The work summarized here provides concise evidence on the likelihood of therapeutic success by targeting alternative NR sites outside the LBP. Furthermore, these approaches may be used not only as a combined therapy but also as a second line of treatment for commercially available drugs that are already exhibiting drug-resistance.

Acknowledgements We are very grateful to Medivation for a research sponsorphip and the Bayer Pharma AG initiative "From Targets to Novel Drugs" Grants4Targets support (2012-05-0708), for the Plan Nacional I+D+i: SAF-2011-29681 Fellowship (MINECO, Gobierno de España).

References

Arnold SF, Notides AC (1995) An antiestrogen: a phosphotyrosyl peptide that blocks dimerization of the human estrogen receptor. Proc Natl Acad Sci U S A 92(16):7475–7479

Arnold LA et al (2006) A high-throughput screening method to identify small molecule inhibitors of thyroid hormone receptor coactivator binding. Sci STKE 2006(341):pl3

Arnold LA et al (2007) Inhibitors of the interaction of a thyroid hormone receptor and coactivators: preliminary structure-activity relationships. J Med Chem 50(22):5269–5280

Askew EB, Minges JT, Hnat AT, Wilson EM (2011) Structural features discriminate androgen receptor N/C terminal and coactivator interactions. Mol Cell Endocrinol 348(2):403–410

Axerio-Cilies, P et al (2011) Inhibitors of androgen receptor activation function-2 (AF2) site identified through virtual screening. J Med Chem 54(18):6197–6205

Becerril J, Hamilton AD (2007) Helix mimetics as inhibitors of the interaction of the estrogen receptor with coactivator peptides. Angew Chem Int Ed Engl 46(24):4471–4473

Biswas A, Mani S, Redinbo MR, Krasowski MD, Li H, Ekins S (2009) Elucidating the 'Jekyll and Hyde' nature of PXR: the case for discovering antagonists or allosteric antagonists. Pharm Res 26(8):1807–1815

Bohl CE et al (2005) Structural basis for accommodation of nonsteroidal ligands in the androgen receptor. J Biol Chem 280(45):37747–37754

Borngraeber S, Budny MJ, Chiellini G, Cunha-Lima ST, Togashi M, Webb P, Baxter JD, Scanlan TS, Fletterick RJ (2003) Ligand selectivity by seeking hydrophobicity in thyroid hormone receptor. Proc Natl Acad Sci U S A 100(26):15358–15363

Brodie J, McEwan IJ (2005) Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. J Mol Endocrinol 34(3):603–615

Buzón V et al (2012) A conserved surface on the ligand binding domain of nuclear receptors for allosteric control. Mol Cell Endocrinol 348(2):394–402

Caboni L et al (2012) "True" antiandrogens-selective non-ligand-binding pocket disruptors of androgen receptor-coactivator interactions: novel tools for prostate cancer. J Med Chem 55(4):1635–1644

Chakraborty S et al (2012) In silico design of peptidic inhibitors targeting estrogen receptor alpha dimer interface. Mol Divers 16(3):441–451

Chakraborty S, Levenson AS, Biswas PK (2013) Structural insights into Resveratrol's antagonist and partial agonist actions on estrogen receptor alpha. BMC Struct Biol 13:27

Chang C et al (1999) Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19(12):8226–8239

Cheng Y Redinbo MR (2011) Activation of the human nuclear xenobiotic receptor PXR by the reverse transcriptase-targeted anti-HIV drug PNU-142721. Protein Sci 20(10):1713–1719

Chrencik JE et al (2005) Structural disorder in the complex of human pregnane X receptor and the macrolide antibiotic rifampicin. Mol Endocrinol 19(5):1125–1134

De Leon JT et al (2011) Targeting the regulation of androgen receptor signaling by the heat shock protein 90 cochaperone FKBP52 in prostate cancer cells. Proc Natl Acad Sci U S A 108(29):11878–11883

Ekins S et al (2007) Human pregnane X receptor antagonists and agonists define molecular requirements for different binding sites. Mol Pharmacol 72(3):592–603

Ekins S et al (2008) Computational discovery of novel low micromolar human pregnane X receptor antagonists. Mol Pharmacol 74(3):662–672

Estébanez-Perpiñá E et al (2005) The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280(9):8060–8068

Estébanez-Perpiñá E et al (2007a) A surface on the androgen receptor that allosterically regulates coactivator binding. Proc Natl Acad Sci U S A 104(41):16074–16079

Estébanez-Perpiñá E, Jouravel N, Fletterick RJ (2007b) Perspectives on designs of antiandrogens for prostate cancer. Expert Opin on Drug Discov 2(10):1341–1355

Estébanez-Perpiñá E et al (2007c) Structural insight into the mode of action of a direct inhibitor of coregulator binding to the thyroid hormone receptor. Mol Endocrinol 21(12):2919–2928

Estébanez-Perpiñá E et al (2007d) A surface on the androgen receptor that allosterically regulates coactivator binding. Proc Natl Acad Sci U S A 104(41):16074–16079

Fuchs S et al (2013) Proline primed helix length as a modulator of the nuclear receptor-coactivator interaction. J Am Chem Soc 135(11):4364–4371

Gearhart MD et al (2003) Monomeric complex of human orphan estrogen related receptor-2 with DNA: a pseudo-dimer interface mediates extended half-site recognition. J Mol Biol 327(4):819–832

Geistlinger TR, Guy RK (2003) Novel selective inhibitors of the interaction of individual nuclear hormone receptors with a mutually shared steroid receptor coactivator 2. J Am Chem Soc 125(23):6852–6853

Gerhard DS et al (2004) The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). Genome Res 14(10B):2121–2127

Grosdidier S et al (2012) Allosteric conversation in the androgen receptor ligand-binding domain surfaces. Mol Endocrinol 26(7):1078–1090

Gunther JR et al (2009a) A set of time-resolved fluorescence resonance energy transfer assays for the discovery of inhibitors of estrogen receptor-coactivator binding. J Biomol Screen 14(2):181–193

Gunther JR, Parent AA, Katzenellenbogen JA (2009b) Alternative inhibition of androgen receptor signaling: peptidomimetic pyrimidines as direct androgen receptor/coactivator disruptors. ACS Chem Biol 4(6):435–440

Haendler B, Cleve A (2012) Recent developments in antiandrogens and selective androgen receptor modulators. Mol Cell Endocrinol 352(1–2):79–91
Harzstark AL, Small EJ (2010) Castrate-resistant prostate cancer: therapeutic strategies. Expert Opin Pharmacother 11(6):937–945
He B, Kemppainen JA, Wilson EM (2000) FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275(30):22986–22994
He B et al The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277(12):10226–10235
Heinlein CA, Chang C (2004) Androgen receptor in prostate cancer. Endocr Rev 25(2):276–308
Huang H et al (2007) Inhibition of drug metabolism by blocking the activation of nuclear receptors by ketoconazole. Oncogene 26(2):258–268
Huggins C (1967) Endocrine-induced regression of cancers. Science 156(3778):1050–1054
Hur E et al (2004) Recognition and accommodation at the androgen receptor coactivator binding interface. PLoS One 2(11):363
Hwang JY et al (2011) Methylsulfonylnitrobenzoates, a new class of irreversible inhibitors of the interaction of the thyroid hormone receptor and its obligate coactivators that functionally antagonizes thyroid hormone. J Biol Chem 286(14):11895–11908
Hwang JY et al (2012) Synthesis and evaluation of sulfonylnitrophenylthiazoles (SNPTs) as thyroid hormone receptor-coactivator interaction inhibitors. J Med Chem 55(5):2301–2310
Irwin JJ, Shoichet BK (2005) ZINC–a free database of commercially available compounds for virtual screening. J Chem Inf Model 45(1):177–182
Irwin JJ et al (2012) ZINC: a free tool to discover chemistry for biology. J Chem Inf Model 52(7):1757–1768
Jensen EV, Khan SA (2004) A two-site model for antiestrogen action. Mech Ageing Dev 125(10–11):679–682
Joseph JD et al (2013) A clinically relevant androgen receptor mutation confers resistance to second-generation antiandrogens enzalutamide and ARN-509. Cancer Discov 3(9):1020–1029
Knudsen KE, Penning TM (2010) Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer. Trends Endocrinol Metab 21(5):315–324
Kojetin DJ et al (2008) Implications of the binding of tamoxifen to the coactivator recognition site of the estrogen receptor. Endocr Relat Cancer 15(4):851–870
Korpal M et al (2013) An F876 L mutation in androgen receptor confers genetic and phenotypic resistance to MDV3100 (enzalutamide). Cancer Discov 3(9):1030–1043
Lack NA et al (2011) Targeting the binding function 3 (BF3) site of the human androgen receptor through virtual screening. J Med Chem 54(24):8563–8573
LaFrate AL et al (2008) Synthesis and biological evaluation of guanylhydrazone coactivator binding inhibitors for the estrogen receptor. Bioorg Med Chem 16(23):10075–10084
LaFrate AL, Carlson KE, Katzenellenbogen JA. (2009) Steroidal bivalent ligands for the estrogen receptor: design, synthesis, characterization and binding affinities. Bioorg Med Chem 17(10):3528–3535
Langley E, Zhou ZX, Wilson EM (1995) Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. J Biol Chem 270(50):29983–29990
Masiello D et al (2002) Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. J Biol Chem 277(29):26321–26326
Mettu NB et al (2007) The nuclear receptor-coactivator interaction surface as a target for peptide antagonists of the peroxisome proliferator-activated receptors. Mol Endocrinol 21(10):2361–2377
Mita Y et al (2010) LXXLL peptide mimetics as inhibitors of the interaction of vitamin D receptor with coactivators. Bioorg Med Chem Lett 20(5):1712–1717
Mohler ML et al (2012) Androgen receptor antagonists: a patent review (2008–2011). Expert Opin Ther Pat 22(5):541–565

Munuganti RS et al (2013) Targeting the binding function 3 (BF3) site of the androgen receptor through virtual screening. 2. development of 2-((2-phenoxyethyl) thio)-1H-benzimidazole derivatives. J Med Chem 56(3):1136–1148.

Nagy L, Schwabe JW (2004) Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci 29(6):317–324

Nandhikonda P et al (2012) Discovery of the first irreversible small molecule inhibitors of the interaction between the vitamin D receptor and coactivators. J Med Chem 55(10):4640–4651

Northrop JP et al (2000) Selection of estrogen receptor beta- and thyroid hormone receptor beta-specific coactivator-mimetic peptides using recombinant peptide libraries. Mol Endocrinol 14(5):605–622

Peer A et al (2014) Comparison of abiraterone acetate versus ketoconazole in patients with metastatic castration resistant prostate cancer refractory to docetaxel. Prostate 74(4):433–440

Phillips C et al (2011) Design and structure of stapled peptides binding to estrogen receptors. J Am Chem Soc 133(25):9696–9699

Ravindranathan P et al (2013) Peptidomimetic targeting of critical androgen receptor-coregulator interactions in prostate cancer. Nat Commun 4:1923

Rodriguez AL, Tamrazi A, Collins ML, Katzenellenbogen JA (2004) Design, synthesis, and in vitro biological evaluation of small molecule inhibitors of estrogen receptor alpha coactivator binding. J Med Chem 47(3):600–611

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95(7):927–937

Singh P, Uzgare A, Litvinov I, Denmeade SR, Isaacs JT (2006) Combinatorial androgen receptor targeted therapy for prostate cancer. Endocr Relat Cancer 13(3):653–666

Souza PC et al (2014) Identification of a new hormone-binding site on the surface of thyroid hormone receptor. Mol Endocrinol 28(4):534–545

Teichert A et al (2009) Quantification of the vitamin D receptor-coregulator interaction. Biochemistry 48(7):1454–1461

Togashi M, Nguyen P, Fletterick R, Baxter JD, Webb P (2005) Rearrangements in Thyroid Hormone Receptor Charge Clusters That Stabilize Bound 3,5',5-Triiodo-L-thyronine and Inhibit Homodimer Formation. J Biol Chem 280(27):25665–25673

Vaz B et al (2009) Computational design, synthesis, and evaluation of miniproteins as androgen receptor coactivator mimics. Chem Commun (Camb), 2009(36):5377–5379

Volakakis N, Malewicz M, Kadkhodai B, Perlmann T, Benoit G (2006) Characterization of the Nurr1 ligand-binding domain co-activator interaction surface. J Mol Endocrinol 37(2):317–326

Wang Y et al (2006a) A second binding site for hydroxytamoxifen within the coactivator-binding groove of estrogen receptor beta. Proc Natl Acad Sci U S A 103(26):9908–9911

Wang L et al (2006b) X-ray crystal structures of the estrogen-related receptor-gamma ligand binding domain in three functional states reveal the molecular basis of small molecule regulation. J Biol Chem 281(49):37773–37781

Wang H et al (2007) Activated pregnenolone X-receptor is a target for ketoconazole and its analogs. Clin Cancer Res 13(8):2488–2495

Wang H et al (2008) The phytoestrogen coumestrol is a naturally occurring antagonist of the human pregnane X receptor. Mol Endocrinol 22(4):838–857

Wang WJ et al (2014) Orphan nuclear receptor TR3 acts in autophagic cell death via mitochondrial signaling pathway. Nat Chem Biol 10(2):133–140

Watkins RE et al (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 292(5525):2329–33

Willson TM, Kliewer SA (2002) PXR, CAR and drug metabolism. Nat Rev Drug Discov 1(4):259–266

Wilson E (2011) Analysis of interdomain interactions of the androgen receptor. Methods Mol Biol 776:113–29

Xue Y et al (2007) Crystal structure of the PXR-T1317 complex provides a scaffold to examine the potential for receptor antagonism. Bioorg Med Chem 15(5):2156–2166

Yuan X et al (2013) Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. Oncogene 33(22):2815–2825
Zhan YY et al (2012) The orphan nuclear receptor Nur77 regulates LKB1 localization and activates AMPK. Nat Chem Biol 8(11):897–904
Zhou HB et al (2007) Bicyclo[2.2.2]octanes: close structural mimics of the nuclear receptor-binding motif of steroid receptor coactivators. Bioorg Med Chem Lett 17(15):4118–4122

Selective Estrogen Receptor Modulators (SERMs) and Selective Androgen Receptor Modulators (SARMs)

Ramesh Narayanan and James T. Dalton

1 Introduction

Innovation and an increase in the fundamental understanding of molecular pharmacology have entirely transformed the pharmaceutical industry and led to the discovery and development of a variety of new therapeutic approaches to disease over the last several decades. The "one size fits all" concept that was prevalent until the late 1990s is being replaced by one centered on personalized medicines and targeted therapeutics, a transformation geared to help physicians treat diseases effectively without unwarranted side effects. Endocrinology and endocrine cancers were the birth place of targeted therapeutics. In 1900, Beatson and Boyd (Boyd 1897, 1899) demonstrated that oophorectomy regressed breast cancer, the first evidence that estrogen is the primary target of breast cancer. This led to subsequent efforts to target breast cancers with anti-estrogenic drugs (Lacassagne 1937). In 1936, Huggins and Hodges demonstrated that orchiectomy or castration (testosterone deprivation) or high dose of estrogens significantly benefited men with prostate cancer (Huggins and Hodges 1941). These findings preceded any knowledge or even discovery of estrogen receptor (ER) or androgen receptor (AR), which were eventually identified in the 1970s and 1980s (Jensen et al. 1969; Kuiper et al. 1996; Chang et al. 1988). The field has grown exponentially since then with the discovery of receptors for other steroid hormones, xenobiotics, bile acids, and other ligands yet to be identified (orphans) (Xie and Evans 2001). Drugs targeting nuclear receptors constitute one of the largest classes, second to those working through G protein coupled receptors (GPCRs). These drugs are used in the treatment or prevention of a plethora of

J. T. Dalton (✉)
Dean and Professor of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan, USA
email: daltonjt@umich.edu

R. Narayanan
Associate Professor, Department of Medicine, Director, Center for Cancer Drug Discover, University of Tennessee Health Science Center, Memphis., USA

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7_11

diseases, ranging from acute inflammation to chronic life threatening diseases such as advanced prostate and breast cancer. Advancements in the knowledge of receptor function, the discovery of coactivators, corepressors, and mapping of global DNA binding elements have all assisted in a better understanding of these proteins and ways to modulate their activity for therapeutic benefit.

2 Estrogen and Androgen Synthesis and Action

Although the ovaries and testes are the predominant sites for estrogen and androgen biosynthesis, in healthy women and men respectively, other tissues and organs contribute to local steroid production (Nelson and Bulun 2001). Several lines of evidence indicate that these hormones are synthesized in the breast, prostate, tumors and other tissues *via* paracrine mechanisms and locally expressed steroidogenic enzymes (Nelson and Bulun 2001; Byrns et al. 2010; Cai et al. 2011; Sasano et al. 2009), questioning the historical dogma of endocrinology and providing further rational for the use and development of selective modulators.

2.1 Steroidogenesis

Androgen and estrogen syntheses are complex and dynamic (Fig. 1). The common precursor for the synthesis of androgens, estrogens, progesterone, and corticosteroids is cholesterol. While the majority of the cholesterol for androgen and estrogen synthesis is obtained from plasma low density lipoprotein (LDL), additional cholesterol is also synthesized *de novo* in the adrenals. Cholesterol is converted to pregnenolone by the mitochondrial cytochrome P450scc enzyme, expressed by the gene Cyp11A1. This enzyme cleaves the side chain carbons 23–27 of cholesterol to create C22 pregnenolone. Pregnenolone is then converted by 17α hydroxylation to 17α hydroxy pregnenolone by 17α hydroxylase enzyme, which is encoded by the gene Cyp 17A1. Deficiencies in this enzyme, expressed in the adrenal cortex, have been associated with pseudohermaphroditism and adrenal hyperplasia (Miller 2012). The first two steps in the steroidogenic pathway are common for the synthesis of androgens, estrogens, corticosteroids, and progesterone. Recently, an inhibitor of Cyp 17A1, abiraterone, was approved by the US FDA to treat advanced prostate cancer. Since the enzyme also mediates the synthesis of corticosteroids, in addition to androgens, glucocorticoids are co-administered with abiraterone to reduce cortisol excess due to the absence of hypothalamus:pituitary:adrenal axis feedback regulation.

All of the subsequent steps in androgen and estrogen synthesis are mediated by a class of enzymes called hydroxysteroid dehydrogenases (HSD). There are two classes of HSDs, namely 3β- and 17β-HSDs, depending on the carbon on which they perform the dehydrogenase activity. These enzymes are involved in the oxidation and reduction of ketone and β-hydroxyl groups at the C3 and C17 positions of androgen and estrogen precursors. The major biological functions of these

17β-HSDs include both biosynthetic activation and inactivation of various estrogens and androgens. Fourteen mammalian 17β-HSDs have been identified to date; and are grouped into oxidative enzymes (17β-HSD types 2, 4, 6, 8, 9, 10, 11, and 14) that catalyze the NAD^+-dependent inactivation of sex hormones and reductive enzymes (17β-HSD types 1, 3, 5, and 7) that catalyze the formation of more potent steroid receptor ligands. The proliferative effects of androgens and estrogens in target tissues and over-expression of 17β-HSDs in cancer have led to intense drug discovery efforts to identify and develop isoform selective 17β-HSD inhibitors that can be used for the treatment of breast, prostate and endometrial cancers, neurological disorders, endometriosis, acne, hirsutism and several other hormone dependent and independent diseases. The final conversion of inactive or less active androgens to active androgens, testosterone and DHT, is mediated by 17β-HSDs and 5α-reductase, respectively. Similarly, conversion of the weak estrogen, estrone, to estradiol is mediated by 17β-HSDs and conversion of testosterone to estradiol is mediated by the enzyme aromatase. Further inactivation of these active androgens and estrogens is mediated by various 17β-HSDs and 3α-HSDs.

The last 5 years have witnessed the discovery of "backdoor pathways" that synthesize active androgens and bypass the conventional routes of biosynthesis (Sharifi and Auchus 2012) (Fig. 1). Although these backdoor pathways make androgens in unconventional ways, they ultimately utilize the same set of enzymes used in traditional steroid biosynthesis in the testes. Estrogen biosynthetic pathways remain unchanged from conventional methods.

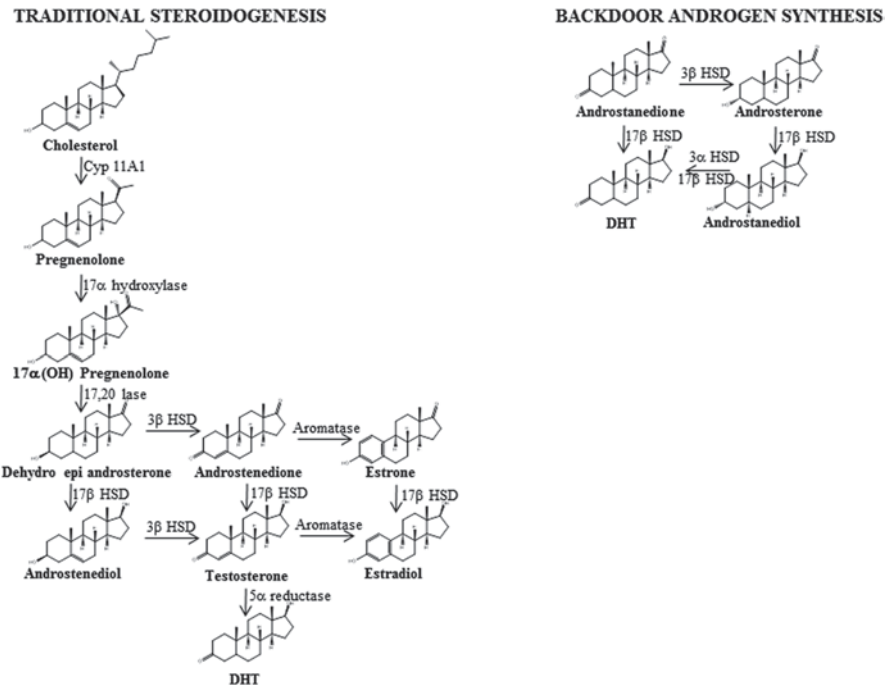


Fig. 1 Steroidogenic pathways (both traditional and backdoor) responsible for the synthesis of androgens and estrogens from cholesterol. HSD-Hydroxysteroid dehydrogenase

2.2 Tissues and Organs Involved in Androgen and Estrogen Biosynthesis

In addition to gonadal synthesis, local steroid biosynthesis produces testosterone and estradiol for the function of the many tissues. Estradiol synthesis in women of reproductive age primarily takes place in the granulosa cells of the ovary, mainly *via* conversion from estrone. In men, 15% of circulating estrogens are synthesized in testes. However, recent evidence suggests that, in post-menopausal women and men undergoing andropause, adipose tissue and skin express steroidogenic enzymes that contribute significantly to local synthesis of these pivotal hormones. Aromatization of testosterone to estradiol plays important roles in estradiol synthesis in peripheral tissues including adipose, skin, brain, bone and others. In post-menopausal women and men, local expression of aromatase is important for synthesis of estradiol (Nelson and Bulun 2001). In post-menopausal women with osteoporosis and dementia, the expression of aromatase is lower in bone and brain, respectively, than that observed in healthy post-menopausal women (Simpson et al. 1997; Jakob et al. 1997; Bulun et al. 1999). Moreover, it was also demonstrated that local expression of aromatase increases with age and obesity, indicating that aromatization becomes primary estradiol synthesis machinery.

Androgen biosynthesis occurs predominantly in the testes. Preclinical studies and human trials indicate that castration leads to over 95% depletion of circulating testosterone (Labrie 2011). The remaining approximately 5% of androgens, which include DHT, testosterone, dehydroepiandrosterone (DHEA), DHEAS, androstenedione, androstenediol, and others, evolve from the adrenals. This finding resulted in the development of treatments (e.g., ketoconazole and abiraterone) for castration-resistant prostate cancers (CRPCs) that block adrenal androgen synthesis. In addition, negligible, but functionally relevant, androgen synthesis also takes place in the peripheral extra-gonadal tissues such as adipose.

2.3 Importance of Estrogens in Development, Physiology, and Pathology

Estrogens are also important for the development and physiology of various tissues. The role of estrogens is mediated by two receptors, ER-α (NR3A1) and ER-β (NR3A2). While ER-α is abundantly found in reproductive tissues, ER-β is expressed widely. Knock-out studies of the ER isoforms and aromatase helped identify the importance of estrogens in variety of physiological functions. These studies also demonstrated the overlapping and distinct functions of the two isoforms in mediating the actions of estrogens. While ER-α is considered a proliferative signal, ER-β is typically considered a repressor of ER-α action and an anti-proliferative isoform. ER-αKO mice were sterile, had high gonadotrophin production, lacked ovulation, had pubertal and mammary development abnormalities, osteoporosis,

and estrogen insensitivity in the uterus. On the other hand, ER-βKO mice phenotypes were mild to moderate and did not or only mildly exhibited many of the above functional abnormalities (Emmen and Korach 2003; Hewitt and Korach 2003). On the other hand, functional abnormalities of ER-βKO mice were exacerbated when challenged with external stimuli such as a high fat diet or inflammatory molecules including lipopolysaccharide (LPS). In addition to these physiological functions, estrogens also play a pivotal role in many diseases, including cancers of the reproductive organs and cardiovascular disease. The physiological and pathological roles of estrogens are summarized in Table 1.

2.4 Importance of Androgens in Development, Physiology, and Pathology

Similar to estrogens in females, androgens are important reproductive hormones in males (Table 1). Androgen insensitivity syndrome and andropause in humans provide unequivocal evidence of the importance of androgens in the development of sexual organs including the penis, scrotum, seminal vesicles, prostate, vas deferens, and epididymis (Zhou 2010). In addition, AR knockout mice exhibit osteoporosis, muscle wasting, insulin resistance and metabolic syndrome, and late onset obesity. Humans with longer poly glutamine repeats in the N-terminal domain of AR develop Kennedy's disease or spinal bulbar muscular atrophy (SBMA), a rare neuromuscular degenerative disease and distinct form of androgen insensitivity syndrome that is characterized by muscle weakness and neuronal deformity.

Table 1 Role of estrogens and androgens

Physiology	Pathology
Estrogens	
Secondary sexual organ development	Endometriosis, Breast: ovary. endometrial, prostate cancers
Bone development and maintenance	Osteoporosis
Brain development and cognition	Alzheimer's, dementia
Glucose and adipose homeostasis	Cardiovascular diseases
Gonadotropin secretion for ovulation	Female pseudohermaphrodit ism and male tall stature with unfused epiphyses
Fusion of epiphysis in men	Gonadotropin feedback irregularity
Androgens	
Secondary sexual organ development	Prostate cancer, sexual dysfunction
Bone development and maintenance	Osteoporosis
Muscle generation and maintenance	Sarcopenia
Brain development and cognition	Dementia, Alzheimer's
Glucose and adipose homeostasis	Cholesterol imbalance, Cardiovascular diseases
Sperm maturation and sexual activity	Gonadotropin feedback irregularity

3 Selective Estrogen Receptor Modulators (SERMs)

The phenotypic and physiological evidence from preclinical animal models and human trials show that ERs are a rational and highly valuable therapeutic target for many conditions. Estradiol was initially proposed as a treatment for post-menopausal osteoporosis and female secondary sexual growth deficiencies. In fact, conjugated estrogen products remain a viable treatment option for many post-menopausal symptoms associated with estrogen deficiency. Shortly after the discovery of ER, estrogen antagonists were suggested as treatments for breast cancer, uterine cancer, and endometrial hyperplasia. However, the ubiquitous expression of ER-α makes it difficult to distinguish between pharmacologic effects in target tissues and off-target effects when treating with estradiol or antagonists. The following scenarios describe the characteristics of an ideal SERM and the various pathophysiological conditions that require tissue-selective ER modulators;

Scenario 1 Post-menopausal women frequently suffer osteoporosis and bone fracture as a result of depleted levels of circulating estrogens. Treating post-menopausal women with estradiol or an ER-α agonist is a viable option therapeutically. However, treating post-menopausal women with estradiol will unfortunately result in ER agonist activity not only in bone but also in breast and uterus, leading to increased incidence of breast and uterine cancers. In this scenario, a small molecule that functions as an agonist in bone, but spares breast, uterus, ovaries and other tissues is considered ideal.

Scenario 2 Hormonal imbalance and other stimuli that hyper-activate ER can result in breast cancer. ER-positive breast cancers are treated with ER antagonists or aromatase inhibitors. However, treating post-menopausal or middle aged women with pure estrogen antagonists results not only in antagonistic activity in breast and regression of breast cancer, but also results in osteoporosis, due to impairment of ER function in bone. In this scenario, a small molecule that functions as an antagonist in breast, but spares bone, uterus, and ovaries is desirable.

Scenario 3 Angiotensin II-induced cardiac hypertrophy and fibrosis as a result of hypertension can be rescued by estradiol through its actions on ER-β (Pedram et al. 2010). However, administration of estradiol will activate not only ER-β and alleviate cardiac hypertrophy and fibrosis, but will also activate ER-α, which can result in breast and endometrial cancers. In this scenario, isoform-selective small molecule agonists that activate ER-β, but do not cross react with ER-α, are highly desirable.

From the above three scenarios, an ideal SERM should be tissue- and isoform-selective and provide therapeutic benefits without associated side effects. Table 2 summarizes the desirable properties of ER ligands in various tissues and the profile for the “Ideal SERM”.

Table 2 Estrogen receptor ligands

Target tissue	Properties
Breast	Antagonist
Endometrium	Antagonist
Platelets	Antagonist
Bone	Agonist
Cholesterol and metabolism	Agonist
Climacteric	Agonist
Vagina	Agonist
Adipose (obesity)	Isoform-selective ERβ agonist
Cardiovascular	ERα antagonist or ERβ selective agonist
CNS	Agonist
Prostate	ERα antagonist or ERβ selective agonist

3.1 Clomiphene

Clomiphene (Fig. 2), belonging to the triphenylethylene structural series, was the first SERM. Its discovery in 1960 preceded the discovery of tamoxifen, the most widely used SERM, by 2 years. Clomiphene is an ER antagonist and the primary choice for ovulatory dysfunction, including polycystic ovary syndrome (PCOS). Ovulatory dysfunction, such as PCOS, luteal phase deficiency and anovulation are

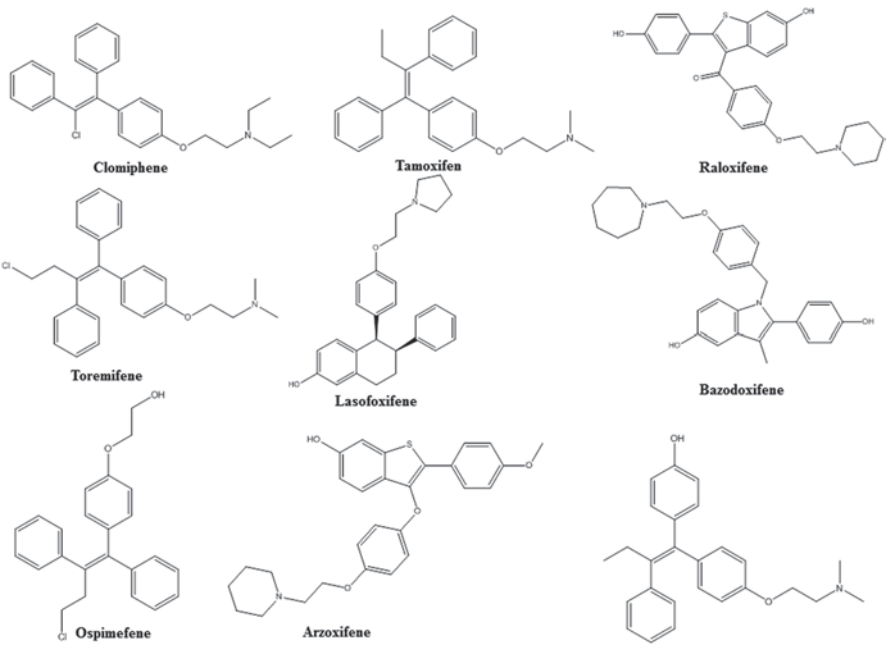


Fig. 2 Selective estrogen receptor modulators (SERMs)

commonly observed reproductive abnormalities in women. As an ER antagonist, clomiphene citrate suppresses the negative feedback inhibition of estradiol and increases secretion of follicle stimulating hormone (FSH), which in turn stimulates follicular development and results in an increase in ovulation rate. Some of the common side effects of clomiphene are hot flashes, abnormal uterine bleeding, nausea and/or vomiting. Although clomiphene increases ovulation by 80–90 % in women with PCOS, it increases pregnancy only by 25–30 %. Endometrial biopsies obtained from women treated with clomiphene indicated that it reduces the thickness of the endometrium, an effect that is more pronounced with clomiphene than other SERMs or aromatase inhibitors.

Clomiphene citrate is also being investigated as a treatment for male hypogonadism. Utilizing the same phenomenon to inhibit the negative feedback loop of gonadotropins, hypo gonadal men treated with clomiphene demonstrated a significant increase in serum testosterone levels and in ADAM scores (Androgen Deficiency in Ageing Male). Interestingly, hypo gonadal men receiving clomiphene for more than 2 years also demonstrated an increase in bone mineral density (BMD) and a decrease in osteoporosis (Moskovic et al. 2012). Data on the changes in BMD in hypo gonadal men and women with PCOS by clomiphene is conflicting. While some studies indicate an increase in BMD with clomiphene, others show that clomiphene might decrease BMD. These seemingly contradictory results could be due to the direct antagonistic effects of clomiphene on ER-α in bone, indirect effects of clomiphene to increase testosterone and estrogens, or differences in the overall hormonal milieu of women and men.

Preclinical studies have indicated that while clomiphene is an antagonist in ovary and endometrium, it is an agonist in the skeletal and cardiovascular systems. Interestingly, while E-clomiphene and Z-chlomiphene both have agonistic activities in bone and cardiovascular tissues, E-chlomiphene is an agonist and Z-chlomiphene is an antagonist in uterus.

3.2 Tamoxifen

Tamoxifen (Fig. 2) was discovered in 1962 by Imperial Chemical Industry (now Astra Zeneca) as a triphenylethylene SERM. Tamoxifen is the most widely used SERM for breast cancer and it functions through its active metabolite, hydroxytamoxifen. Tamoxifen has become the most widely studied SERM from the mechanistic perspective as it functions as an ER antagonist in breast, but agonist in uterus and endometrium. Tamoxifen binds to ER with a high affinity of 2 nM and antagonizes both isoforms of ER.

Tamoxifen is currently in use for node-negative breast cancer in pre- and postmenopausal women and for node-positive breast cancer in post-menopausal women. It is also effective in metastatic breast cancer and has recently been approved for the prevention of breast cancer in high risk women. Multiple clinical trials have demonstrated significant survival benefits in women with breast cancer treated with

tamoxifen (Davies et al. 2013; Darby et al. 2011). Being a SERM, prolonged use of tamoxifen provided beneficial anti-cancer effects in the breast and also modestly preserved BMD and decreased the number of fractures (Ryan et al. 1991; Love et al. 1992). Since post-menopausal women undergoing ER-antagonistic treatments have a higher incidence of osteoporosis and fractures, tamoxifen's small, but beneficial, effects on bone are considered one of the advantages for improved survival of these patients. Recently, tamoxifen also received attention for its potential for favorable effects on cardiovascular diseases (Love et al. 1994). A number of cardiovascular diseases markers such as LDL, HDL, and triglycerides are favorably altered by tamoxifen, making it potentially more valuable for patients with breast cancer.

Despite its many beneficial effects, one major concern with the use of tamoxifen is its effects on the endometrium. Endometrial cancer is increased by 2–4 fold in randomized trials (Chen et al. 2014). Uterine-related symptoms include vaginal bleeding and leucorrhoea. Multiple lines of evidence suggest that while tamoxifen functions as an ER antagonist in breast, it functions as an agonist in the uterus and endometrium (Wood et al. 2010). This warrants careful monitoring of breast cancer patients receiving tamoxifen for uterine cancer.

3.3 Raloxifene

Raloxifene (Fig. 2) belonging to the benzothiophene structural series was discovered and developed by Eli Lilly for post-menopausal osteoporosis. Raloxifene provides unique SERM properties with one of the best profiles known. While raloxifene functions as an ER agonist in bone and in the regulation of serum lipids, it functions as an antagonist in breast and uterus, making it a highly valuable tool to combat post-menopausal osteoporosis without the disadvantages of activating ER in other tissues (D'Amelio and Isaia 2013). Studies on the effects of raloxifene on bone turnover markers indicated that raloxifene prevented bone loss and fractures at multiple sites with concurrent increase in BMD (D'Amelio and Isaia 2013). Bone turnover markers were significantly reduced and bone formations markers were upregulated by raloxifene.

Recent evidence suggests that raloxifene significantly prevented breast cancer incidence in post-menopausal women, with results comparable to that of tamoxifen (Vogel et al. 2006). Women treated with raloxifene demonstrated a breast cancer incidence of only 1.7 per 1000, whereas placebo-treated women had a breast cancer incidence of 3.7 per 1000. The outcome of the Study of Tamoxifen and Raloxifene (STAR) trial indicated that raloxifene was as effective as tamoxifen in reducing invasive breast cancer without any thromboembolic side effects (Freedman et al. 2011). While raloxifene had a better benefit/risk ratio than tamoxifen in post-menopausal women with a uterus, raloxifene and tamoxifen had comparable benefit/risk ratios in post-menopausal women who had undergone hysterectomy. Overall, the clinical data indicates that raloxifene has a better benefit/risk ratio than tamoxifen.

Raloxifene lowered LDL cholesterol without affecting HDL cholesterol, suggesting that it might also have beneficial effects on these markers of cardiovascular disease (Francucci et al. 2005). Raloxifene also showed favorable effects on CNS function, including effects on climacteric symptoms such as hot flashes. Among all the commercially available first-generation SERMs, raloxifene is considered by many to have a near-perfect SERM profile, with favorable benefits in desired organs and a lack of unwarranted side effects.

3.4 Toremifene

Toremifene (Fig. 2) differs from tamoxifen by only one chlorine atom and possess properties comparable to that of tamoxifen (Buzdar and Hortobagyi 1998). Toremifene is also used for the treatment of advanced breast cancer. While toremifene functions as an ER antagonist in breast, it functions as an agonist with regard to bone, lipids, and uterus. Toremifene was also tested as a treatment for osteoporosis in men undergoing androgen deprivation therapy (ADT) (Smith et al. 2011). Although it protected bone loss in men undergoing ADT, the studies necessary to establish its efficacy and safety in this population were never completed. Similar to tamoxifen, toremifene's active N-desmethyl and 4-hydroxylated metabolites bind to and antagonize ER more potently than the parent molecule.

3.5 Second Generation SERMs

Several SERMS such as lasofoxifene, arzoxifene, ospemifene, afimoxifene, arzoxifene, and bazodoxifene (Fig. 2) have been approved since 2000 (Komm and Chines 2012). All of these SERMs offer distinct tissue- selective properties and an unique profile compared to the first generation SERMs described above. While lasofoxifene has ER agonistic activity in bone, it has antagonistic activity in breast and uterus making it suitable for the treatment of osteoporosis, breast cancer, and vaginal atrophy. Phase III trials with lasofoxifene showed that it increased BMD significantly without any associated endometrial or thromboembolic effects (Malozowski 2010). In other trials, lasofoxifene improved vaginal atrophy, reduced ER-positive breast cancer growth and coronary disease risk (LaCroix et al. 2010; Ensrud et al. 2010; Goldstein et al. 2011). Similarly, bazodoxifene reduced the incidence of osteoporosis in post-menopausal women without causing an increase in endometrial hyperplasia (Miller et al. 2008). As second generation SERMs, these compounds have better safety profiles and exhibit near ideal SERM properties such as ER antagonistic effects in breast and uterus and agonistic properties in bone, blood and CNS, making them more broadly applicable to many women's health issues.

3.6 ER-β Selective Ligands

The effects of estrogens are mediated by two isoforms of ER, ER-α and ER-β. Although the LBD of the two isoforms is only 59% homologous, the ligand binding pocket (LBP) differs only by two amino acids, making them highly similar (Katzenellenbogen 2011). Despite this striking similarity, academic investigators and pharmaceutical companies have successfully developed ligands that bind preferentially to one or the other isoforms in order to take advantage of the beneficial effects of the individual isoforms without impinging on the function of the other isoform. Several structurally diverse molecules have been identified that bind to ER-β at low nanomolar to picomolar affinity, without cross reacting with ER-α (Fig. 3).

Diverse pharmacophores ranging from a simplistic linear diphenolic compound that achieves an inter-phenolic distance similar to estradiol to highly complex structures have been identified (Minutolo et al. 2011). The most common ER-β agonist template configuration is a (hetero) bicyclic compound substituted with a phenyl side chain such as genistein. Other templates include carbaldehyde oxime derivatives (Wyeth), hydroxyl-biphenyl carbaldehyde oximes (Wyeth), diaryl substituted salicylaldoximes (UIUC), alkyl linked (UIUC), cycloalkanes-linked biphenyls (Acadia), spiro indene-indenes (Merck) and many more. Academic and industrial groups have patented close to 50 different structural templates as ER-β ligands. These templates represent a heterogeneous mixture with regard to their level of characterization and degree of design success. Although several groups have been successful in discovering and developing high affinity ER-β-selective templates, Wyeth (now Pfizer) stands out as the leader in the field in terms of attaining extremely high affinity ligands and exploring a wide variety of templates. Further, Wyeth is the only pharmaceutical company to advance a synthetic ER-β agonist, prinaberel (ERB-041), to clinical trials. They explored a variety of clinical indications, including rheumatoid arthritis, Crohn's disease, and endometriosis. However,

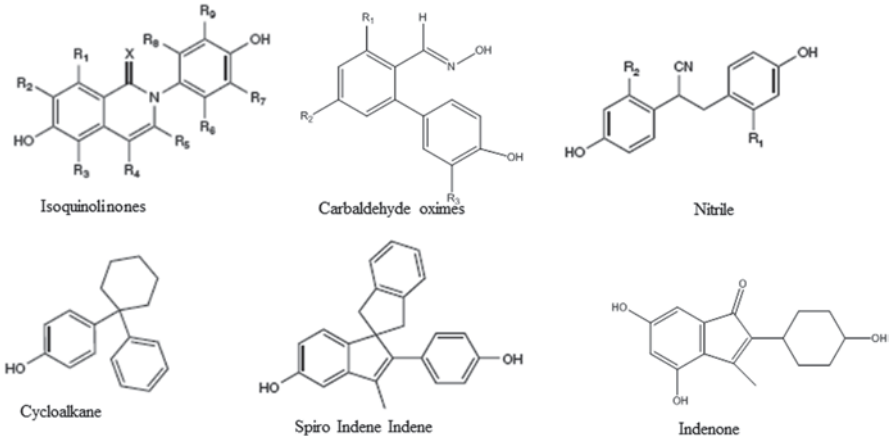


Fig. 3 Estrogen receptor β selective ligands

the data were neither convincing nor published in entirety. The only other clinical candidate ER-β agonist, MF-101 (Bionovo) is an herbal extract containing a combination of ER-β agonists of plant origin. However their recently reported clinical trial results were only marginally successful in suppressing post-menopausal hot flashes. Diarylpropionitrile (DPN), which was initially developed by Katzenellenbogen et al., has been used extensively for academic exploration of the role of ER-β (Meyers et al. 2001).

ER-β agonists have been highly sought agents following the discovery of ER-β in 1996 (Kuiper et al. 1996). The distinct tissue-distribution alone seemed to suggest that tissue-selective estrogenicity was possible, which is further supported by the diverse and often opposing functional roles of ER-β, compared to ER-α. The theoretical problem of designing ER-β-selective agents was fully defined following the first crystal structure in 1999 of genistein bound to the ER-β LBD. Combined with the earlier ER-β LBD structures, this work revealed that the polar interactions available within the ER-α and ER-β ligand binding pockets are identical. Importantly, the ER-α and ER-β ligand binding pockets only varied by two amino acids, M336/L384 and I373/M421, producing topological and pocket size differences that necessarily must be the basis for subtype selectivity.

Multiple ER-β selective chemotypes with promising preclinical activity exist across a very broad chemical space. Many of these molecules have demonstrated promise as potential treatments for inflammation (Wyeth), cardiovascular diseases, obesity, and metabolic diseases (GTx), hormone therapy (Wyeth and Bionovo for endometriosis and post-menopausal symptoms) and prostatic disease (Eli Lilly). However, only a small fraction of the theoretically useful indications have been explored clinically, leaving room for exploration of many clinical indications including anxiety or depression, colon cancer, hepatic fibrosis, memory, neuroprotection, neuropathic or inflammatory pain, and wound healing, to name just a few.

Further clinical testing is needed to better understand the beneficial effects and liabilities of ER-β agonists in man. Nonetheless, the breadth and depth of ER-β agonists suggest that the preclinical pipeline is robust. Even in the absence of a clear clinical proof-of-principle, the prospects for ER-β agonist development seem promising.

4 Selective Androgen Receptor Modulators

Androgen Receptor is widely expressed in the body, and steroidal androgens, the important male circulating hormones, play important roles in maintaining bone strength, increasing muscle mass, and promoting the development of secondary sexual characteristics. However, virilizing side effects and limitations with regard to their route of administration have limited the clinical use of testosterone and closely related steroidal analogues and spurred interest in the development of SARMs. Testosterone preparations currently available in the US are administered either transdermally or intramuscularly. Transdermal preparations may cause skin

sensitivity or transference of the androgen to individuals other than the patient (Wilson et al. 1998). Intramuscular testosterone often provides supraphysiologic levels of testosterone of unknown safety significance (Dhar et al. 2005). Orally available steroid androgens may pose a risk of hepatotoxicity (Boada et al. 1999).

In addition to the recognized risks of testosterone therapy including acne, edema, dyslipidemia, benign prostatic hypertrophy, and worsening of sleep apnea, the major concerns remain to be virilization in females, prostate cancer and cardiovascular toxicity in males (Osterberg et al. 2014; Vigen et al. 2013; Tan and Teoh 2013). The putative beneficial effects of testosterone therapy in female and certain male populations appear to be outweighed by the risks of virilization and unknown cardiovascular effects. The beneficial effects and success of commercially available SERMs have created interest to develop a therapeutic paradigm using tissue-specific SARMs that may act as full or partial agonists and antagonists for the AR in different tissues.

SARMs can be divided into four categories based on the structure of their pharmacologically active portions: aryl-propionamides, bicyclic hydantions, quinolines, and tetrahydroquinolones (Fig. 4). The pharmacokinetics and AR specificities of the compound in each of these categories differ widely (Gao et al. 2006). These two qualities, along with their tissue selectivity may give SARMs therapeutic advantages over steroidal androgens and create the possibility for SARMs targeting specific disease states or tissues.

The tissue selectivity of a SARM in preclinical studies is typically evaluated in the Hershberger assay, where anabolic activity on muscle is compared to androgenic effects on prostate and seminal vesicles. While steroidal androgens such as DHT or testosterone non-selectively increase the size and weight of both muscle and prostate or seminal vesicles of castrated rats, SARMs only increase the muscle mass, while sparing prostate and seminal vesicles (Yin et al. 2003; Kim et al. 2005; Schlienger et al. 2009; Nique et al. 2012; Nagata et al. 2014). The same phenomenon is expected in humans too, where SARMs have been shown to increase lean body mass and physical function without affecting serum levels of prostate specific

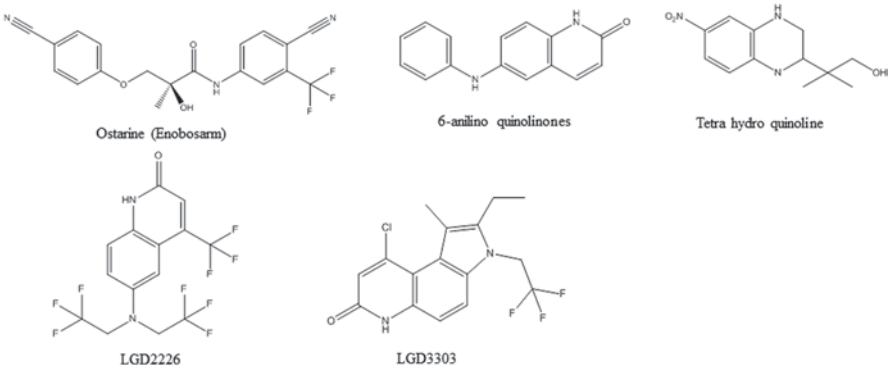


Fig. 4 Selective androgen receptor modulators (SARMs)

antigen (PSA) in men or causing hair-growth or other signs of virilization in women (Dalton et al. 2013; Dobs et al. 2013).

The field of SARMs is at an early stage, as none have been approved for clinical use. However, several companies have reported the results of preclinical and clinical studies with SARMs. The various pharmacophores, along with their demonstrated activity and potential clinical utility, are discussed below.

4.1 GTx Inc.

GTx, Inc. has published extensive results on the preclinical pharmacology and potential therapeutic applications of aryl propionamide SARMs. Enobosarm (Fig. 4; also referred to as S-22, GTx-024 and ostarine in the literature), their lead aryl propionamide SARM, is the most advanced clinical candidate. Enobosarm demonstrated exciting data in proof-of-concept Phase II clinical trials. A phase II double blind, randomized, placebo-controlled trial in elderly men and postmenopausal women and a phase II, double blind, randomized, placebo-controlled trial in men and women suffering from cancer cachexia have been reported (Dalton et al. 2013; Dobs et al. 2013). Without a prescribed diet or exercise regimen, all subjects treated with enobosarm had a dose-dependent increase in total lean body mass (LBM), with the 3 mg/day cohort achieving an increase of more than 1 kg compared to baseline and placebo after 3 months of treatment. Treatment with enobosarm also resulted in a dose-dependent improvement in functional performance measured by a stair climb test, with the 3 mg/day cohort achieving clinically significant improvement in speed and power. The SARM also demonstrated a favorable safety profile with no serious adverse events reported and no clinically significant changes in measurements for serum PSA (prostate), sebum production (skin and hair), or serum LH (pituitary) compared to placebo. Interestingly, subjects treated with 3 mg/day of enobosarm had a decline in fasting blood glucose, reduction in insulin levels, and reduction in insulin resistance (homeostasis model assessment) as compared to baseline, suggesting that SARMs might have therapeutic potential in diabetics or people at risk for diabetes. Phase I clinical studies with enobosarm showed that it was rapidly absorbed after oral administration with a half-life of about 1 day. Phase III trials to examine the safety and efficacy of enobosarm to prevent and treat muscle wasting in patients with stage III or IV non-small cell lung cancer were completed in 2013, and will be the subject of forthcoming reports by the investigators. Preliminary reports suggest that enobosarm was very well tolerated and demonstrated promising efficacy on LBM, but produced mixed results on stair climb power depending on the type of chemotherapy that the patients received.

4.2 Ligand Pharmaceuticals

Ligand Pharmaceuticals' SARM program was based on a quinolinones structure. Clinical candidates from Ligand have included bicyclic 6-anilino quinolinones

that demonstrated tissue-selective full myoanabolic activity (Vajda et al. 2009; van Oeveren et al. 2006). Ligand chose LGD2226 as its first pre-clinical lead compound (Wang et al. 2006). LGD2226 was weaker than testosterone, but demonstrated myoanabolism and osteoanabolism, as evidenced by increases in BMD, improvements in bone structure and strength, and positive effects on bone biomarkers.

Ligand conducted Phase I clinical trials for frailty and osteoporosis with LGD2941 in collaboration with TAP Pharmaceuticals (Zhang et al. 2009). A publication characterized the pre-clinical osteo- and myoanabolic properties of LGD2941 in rats. LGD2941 demonstrated improved bioavailability relative to LGD2226 while maintaining hypermyoanabolic and hyperosteoanabolic properties in male and female *in vivo* maintenance models. The myoanabolism was seen as 180% and 100% of levator ani weight retention at 10 and 1 mg/kg, respectively, compared with 100% and 50% of ventral prostate weight retention at the same doses (Martinborough et al. 2007). The osteoanabolism was seen as a small increase in lumbar space compression strength and larger increases in femur bending strength, indicating effectiveness in cancellous and cortical bone, respectively (Miner et al. 2007; Bhasin and Jasuja 2009).

A third compound, LGD3303, demonstrated hypermyoanabolic and osteoanabolic agonist in rats with an E_{max} of 220% in levator ani but also supported prostate weight at this dose (Kudwa et al. 2010). Ligand performed a pre-clinical bone characterization in a post-menopausal rat model (ovaries removed followed by 8 week waiting period before a 12 week treatment period) that demonstrated improvements in BMD (0.19 g/cm^2 for LGD-3303 vs. 0.175 g/cm^2 for control), femur mechanical strength (230 N for LGD3303 vs. 190 N for control), and trabecular bone volume (14% for LGD3303 vs. 10% for control) compared to untreated ovariectomized control (Kudwa et al. 2010). LGD3303 alone did not fully recover BMD or trabecular volume as compared with sham operated intact females. However, there is no evidence indicating that this SARM was advanced to clinical development.

4.3 Merck

Merck published data and results on a variety of steroidal SARMs, which were variations of the 4-azasteroidal template of finasteride, a 5α-reductase inhibitor (Schmidt et al. 2009). Modifications at several positions reportedly produce tissue-selective activity, with agonist activity in bone and muscle and antagonist activity in prostate or uterus. A series of 17-hydroxy-4-azasteroids was analyzed in an *in vitro* transactivation assay to select the best azasteroid, which was demonstrated to be osteoanabolic in an *in vivo* bone formation rate of 82% of DHT at 3 mg/kg, but with low virilization potential (only 1% of uterine weight) in a 24 day *in vivo* ovariectomized rat model.

Merck also patented two distinct diaryl SARM templates. The diaryl butanamides closely resemble the propionanilides. Unfortunately, Merck appears to have discontinued its SARM program in 2011–2012. In addition to the above companies and structural series, Bristol Myers Squibb, Eli Lilly, Johnson and Johnson, Kaken,

Pfizer and several others have SARM programs that are either in late stage preclinical development or early clinical trials.

4.4 Disease Targets for SARMs

SARMs have the potential to treat many diseases that are associated with muscle and bone loss. Although testosterone could in theory be used as a treatment for such conditions, concerns related to cardiovascular risk, prostate cancer and virilization that are associated with it have limited its use for anything other than male hypogonadism.

Muscle Wasting Disease states that result in rapid loss of muscle are considered as high priority indications for SARMs. Cachexia is a multifactorial syndrome that often occurs in patients with cancer, kidney disease, sepsis, and burns and is characterized by weight loss, muscle wasting, declines in physical function, and decreases in appetite. Elevated levels of cytokines, namely IL-6, TNF-α, TFN-1β, IFN-γ, and proteolysis inducing factor, are thought to contribute to inflammation and wasting (Tsoli and Robertson 2013; Argiles et al. 2005). Muscle wasting is a serious cancer-related symptom that occurs early in the course of the disease, with the highest prevalence in gastrointestinal, pancreatic and lung cancer (Bruera 1997; Dewys et al. 1980). Muscle wasting as evidenced by loss of lean body mass, is strongly associated with poor prognosis and shorter survival (Prado et al. 2008; Martin et al. 2013; Tan et al. 2009) regardless of body weight. The potential of a SARM to improve or prevent the loss of lean body mass may therefore provide a therapeutic approach to decrease the morbidity and mortality associated with cachexia.

Sarcopenia Age-related decline in lean body mass results in the clinical condition known as sarcopenia in older individuals. An increase in the elderly population has contributed to the growing number of frail men and women that are unable to carry out activities of daily living and are thus in need of assisted-care. While enhancing protein intake and exercise programs offer means to combat the muscle loss that occurs with aging, hormonal therapy is likely to show more pronounced effects. An agent capable of selectively increasing muscle performance without androgenic side effects such as prostate growth in men and virilization in women (side effects of steroidal androgens) is desirable for the treatment of sarcopenia. Phase II trials with enobosarm showed significant improvements in the ability of healthy elderly men and women to climb stairs accompanied by significant increases in lean body mass and decreases in fat mass after only 86 days. Lack of PSA increases in men and hair growth in women further corroborated enobosarm's selective anabolic effects. Thus, clinical proof of the benefits of SARM treatment for improving strength exists and shows promising for treating age-related decline in muscle strength.

Breast Cancer While breast cancer is molecularly classified based on three therapeutic targets, ER, PR, and HER2 (Podo et al. 2010), AR-positive breast cancers are becoming the focus for several groups. While ER, PR, and HER2 are oncogenic

in breast cancer, AR has historically been considered anti-proliferative and beneficial (Peters et al. 2012). Until the 1970s, breast cancer was treated mostly with non-aromatizable androgens such as dihydrotestosterone or fluoxymesterone (Kennedy 1958; Adair and Herrmann 1946). AR is highly expressed in breast cancer with more than 75–95 % of ER-positive and 40–70 % of ER-negative breast cancers expressing AR (Garay and Park 2012; Niemeier et al. 2010; Narita et al. 2006). However, virilizing side effects and fears of aromatization to estrogens have limited the use of steroidal androgens in breast cancer. SARMs due to their tissue selectivity, lack of virilizing side effects, and ability to extend androgen therapy to women could become a valuable therapeutic tool for breast cancer. Enobosarm is currently in a Phase II clinical trial to treat women with ER- and AR- positive breast cancer.

5 Mechanisms for Tissue Selectivity of SERMs and SARMs

Several plausible mechanisms for the tissue selectivity of the SERMs and SARMs have been proposed (Smith and O'Malley 2004; McDonnell and Wardell 2010; Narayanan et al. 2008; Bohl et al. 2005a). The most convincing of them are ligand-induced conformational changes in the receptor, the varied expression of coactivators and corepressors in different tissues, altered cell signaling, and other post translational modifications of the receptor and coactivators.

A. Structural modification with different ligands

It is hypothesized that individual SERMs and SARMs may induce specific and unique changes in receptor conformation, which accounts for their particular pharmacological properties in target tissues. The three dimensional structure of various receptors, including the AR and ER in the presence or absence of ligands, has been determined (Bohl et al. 2005a, b; Wu et al. 2005). This provides a good understanding of the mechanism involved in the ligand binding and in the transcription of the target genes. The LBD of the ER and AR has 12 helices, which form a compact structure and the ligand binding pocket. The orientation of helix 12 (H12), a very important helix for ligand binding, is a consequence of allosteric effects induced by the particular chemical structure of the specific ligand. In the absence of a ligand, the LBD of AR and ER is bound to chaperones or corepressors which recruit transcriptional complexes that recruit histone deacetylases (HDAC). These HDACs generate a heterochromatin. The corepressors contain corepressor nuclear-receptor box (CoRNR box), which docks to a hydrophobic groove in the surface of the LBD containing the H3 and H4. In most cases, an agonist promotes complex allosteric effects that lead to an alternative positioning of H12 on the LBD core, which disrupts the hydrophobic groove and leads to corepressor complex dissociation (Heldring et al. 2007). AR and ER LBD were crystallized with steroidal and non-steroidal ligands, revealing differences in their crystal structure (Bohl et al. 2005a, b; Wu et al. 2005; Sack et al. 2001; Brzozowski et al. 1997). The crystal structure of the AR LBD in the presence of DHT shows that hydrophobic interactions and hydrogen

bonding play critical roles in ligand binding (Sack et al. 2001). The 3-keto group on ring A forms hydrogen bonds with the side chains of residues Q711 and R752 directly or indirectly, ring C is in close contact with the main chain of L704 and side chain of N705, and the 17-β-OH group H bonds with the side chains of N705, and T877. The interaction between the ligand and the residues N705 and T877 is very crucial to the conformational changes. On the other hand, the crystal structure with bicalutamide is different from the DHT- induced conformation (Bohl et al. 2005a). Such differences were also true for ER in the presence of SERMs (Wu et al. 2005; Brzozowski et al. 1997). These structural differences could possibly result in differential effects of SERMs and SARMs in different tissues. These differences in conformation in the presence of different ligands also arise from the milieu of various receptor interacting proteins such as coactivators and corepressors (Heldring et al. 2007).

B. Coregulator levels in target tissues

RU486 functions as a partial agonist of PR and GR *in vitro* depending on the cellular environment. In T47D breast cancer cells, RU486 functions as an antagonist and in HeLa, cervical cancer cells, it functions as an agonist (Smith and O'Malley 2004). It was shown that this cell type specific action on transcription correlates with the ratio of coactivator/corepressor in these cells (Smith and O'Malley 2004). Similarly, over expression of SMRT, a corepressor, in cells convert the partial agonist Cyproterone Acetate (CPA) to an antagonist. This indicates that in tissues where the coactivator levels are higher, a ligand can exhibit agonistic activity, but that it may act as an antagonist in tissues with lower coactivator levels. To date, more than 200 coactivator proteins have been identified that bind and activate AR or ER (www.nursa.org). The AR and ER coactivator expression and interaction were found to be different in different tissues (Smith and O'Malley 2004; Muller et al. 2000).

C. Influence of cell signaling pathways on tissue selectivity

Intracellular signaling pathways also play an important role in the function of steroid receptors. The modulation of kinases and phosphatases in cells can lead to increases or decreases in the phosphorylation status of receptors and coregulators, which in turn will lead to alteration in the effects elicited by a tissue selective receptor modulator (Narayanan et al. 2008).

6 Future

Both SERMs and SARMs have broad utility for the prevention and treatment of disease. Although SERMs have been used clinically for more than half-a-century, isoform-selective agents, especially ER-β-selective ligands, stand poised to have a significant impact on cardiovascular and metabolic disease. Likewise, SARMs are just reaching the clinic, with several pharmaceutical companies racing to advance one to therapeutic use in cancer cachexia, sarcopenia, or other muscle conditions. The medical literature provides ample evidence that patents with greater muscle

(lean body) mass survive longer (von Haehling and Anker 2012). The ability of SARMs to improve lean body mass and physical function suggest that they, like SERMs, will eventually constitute one of tools in the armamentarium to combat a variety of conditions.

References

Adair FE, Herrmann JB (1946) The use of testosterone propionate in the treatment of advanced carcinoma of the breast. Ann Surg 123:1023–1035

Argiles JM, Busquets S, Felipe A, Lopez-Soriano FJ (2005) Molecular mechanisms involved in muscle wasting in cancer and ageing: cachexia versus sarcopenia. Int J Biochem Cell Biol 37:1084–1104

Bhasin S, Jasuja R (2009) Selective androgen receptor modulators as function promoting therapies. Curr Opin Clin Nutr Metab Care 12:232–240

Boada LD, Zumbado M, Torres S, Lopez A, Diaz-Chico BN et al (1999) Evaluation of acute and chronic hepatotoxic effects exerted by anabolic-androgenic steroid stanozolol in adult male rats. Arch Toxicol 73:465–472

Bohl CE, Gao W, Miller DD, Bell CE, Dalton JT (2005a) Structural basis for antagonism and resistance of bicalutamide in prostate cancer. Proc Natl Acad Sci U S A 102:6201–6206

Bohl CE, Miller DD, Chen J, Bell CE, Dalton JT (2005b) Structural basis for accommodation of nonsteroidal ligands in the androgen receptor. J Biol Chem 280:37747–37754

Boyd S (1897) On oophorectomy in the treatment of cancer. Br Med J 2:890–896

Boyd S (1899) Remarks on oophorectomy in the treatment of cancer of the breast. Br Med J 1:257–262

Bruera E (1997) ABC of palliative care. Anorexia, cachexia, and nutrition. BMJ 315:1219–1222

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758

Bulun SE, Zeitoun K, Sasano H, Simpson ER (1999) Aromatase in aging women. Semin Reprod Endocrinol 17:349–358

Buzdar AU, Hortobagyi GN (1998) Tamoxifen and toremifene in breast cancer: comparison of safety and efficacy. J Clin Oncol 16:348–353

Byrns MC, Duan L, Lee SH, Blair IA, Penning TM (2010) Aldo-keto reductase 1C3 expression in MCF-7 cells reveals roles in steroid hormone and prostaglandin metabolism that may explain its over-expression in breast cancer. J Steroid Biochem Mol Biol 118:177–187

Cai C, Chen S, Ng P, Bubley GJ, Nelson PS et al (2011) Intratumoral de novo steroid synthesis activates androgen receptor in castration-resistant prostate cancer and is upregulated by treatment with CYP17A1 inhibitors. Cancer Res 71:6503–6513

Chang CS, Kokontis J, Liao ST (1988) Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 240:324–326

Chen JY, Kuo SJ, Liaw YP, Avital I, Stojadinovic A et al (2014) Endometrial cancer incidence in breast cancer patients correlating with age and duration of tamoxifen use: a population based study. J Cancer 5:151–155

Dalton JT, Taylor RP, Mohler ML, Steiner MS (2013) Selective androgen receptor modulators for the prevention and treatment of muscle wasting associated with cancer. Curr Opin Support Palliat Care 7:345–351

D'Amelio P, Isaia GC (2013) The use of raloxifene in osteoporosis treatment. Expert Opin Pharmacother 14:949–956

Darby S, McGale P, Correa C, Taylor C, Arriagada R et al (2011) Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. The Lancet 378:1707–1716

Davies C, Pan H, Godwin J, Gray R, Arriagada R et al (2013) Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 381:805–816

Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM et al (1980) Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. Am J Med 69:491–497

Dhar R, Stout CW, Link MS, Homoud MK, Weinstock J et al (2005) Cardiovascular toxicities of performance-enhancing substances in sports. Mayo Clin Proc 80:1307–1315

Dobs AS, Boccia RV, Croot CC, Gabrail NY, Dalton JT et al (2013) Effects of enobosarm on muscle wasting and physical function in patients with cancer: a double-blind, randomised controlled phase 2 trial. Lancet Oncol 14:335–345

Emmen JM, Korach KS (2003) Estrogen receptor knockout mice: phenotypes in the female reproductive tract. Gynecol Endocrinol 17:169–176

Ensrud K, LaCroix A, Thompson JR, Thompson DD, Eastell R et al (2010) Lasofoxifene and cardiovascular events in postmenopausal women with osteoporosis: Five-year results from the Postmenopausal Evaluation and Risk Reduction with Lasofoxifene (PEARL) trial. Circulation 122:1716–1724

Francucci CM, Romagni P, Boscaro M (2005) Raloxifene: bone and cardiovascular effects. J Endocrinol Invest 28:85–89

Freedman AN, Yu B, Gail MH, Costantino JP, Graubard BI et al (2011) Benefit/risk assessment for breast cancer chemoprevention with raloxifene or tamoxifen for women age 50 years or older. J Clin Oncol 29:2327–2333

Gao W, Kim J, Dalton JT (2006) Pharmacokinetics and pharmacodynamics of nonsteroidal androgen receptor ligands. Pharm Res 23:1641–1658

Garay JP, Park BH (2012) Androgen receptor as a targeted therapy for breast cancer. Am J Cancer Res 2:434–445

Goldstein SR, Neven P, Cummings S, Colgan T, Runowicz CD et al (2011) Postmenopausal Evaluation and Risk Reduction With Lasofoxifene (PEARL) trial: 5-year gynecological outcomes. Menopause 18:17–22

Heldring N, Pawson T, McDonnell D, Treuter E, Gustafsson JA et al (2007) Structural insights into corepressor recognition by antagonist-bound estrogen receptors. J Biol Chem 282:10449–10455

Hewitt SC, Korach KS (2003) Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. Reproduction 125:143–149

Huggins CSR, Hodges CV (1941) Studies on prostatic cancer: II. The effects of castration on advanced carcinoma of the prostate gland. Arch Surg 43:209–223

Jakob F, Siggelkow H, Homann D, Kohrle J, Adamski J et al (1997) Local estradiol metabolism in osteoblast- and osteoclast-like cells. J Steroid Biochem Mol Biol 61:167–174

Jensen EV, Suzuki T, Numata M, Smith S, DeSombre ER (1969) Estrogen-binding substances of target tissues. Steroids 13:417–427

Katzenellenbogen JA (2011) The 2010 Philip S. Portoghese Medicinal Chemistry Lectureship: addressing the "core issue" in the design of estrogen receptor ligands. J Med Chem 54:5271–5282

Kennedy BJ (1958) Fluoxymesterone therapy in advanced breast cancer. N Engl J Med 259:673–675

Kim J, Wu D, Hwang DJ, Miller DD, Dalton JT (2005) The para substituent of S-3-(phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamid es is a major structural determinant of in vivo disposition and activity of selective androgen receptor modulators. J Pharmacol Exp Ther 315:230–239

Komm BS, Chines AA (2012) An update on selective estrogen receptor modulators for the prevention and treatment of osteoporosis. Maturitas 71:221–226

Kudwa AE, Lopez FJ, McGivern RF, Handa RJ (2010) A selective androgen receptor modulator enhances male-directed sexual preference, proceptive behavior, and lordosis behavior in sexually experienced, but not sexually naive, female rats. Endocrinology 151:2659–2668

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925–5930
Labrie F (2011) Blockade of testicular and adrenal androgens in prostate cancer treatment. Nat Rev Urol 8:73–85
Lacassagne A (1937) The relation between hormones and cancer. Can Med Assoc J 37:112–117
LaCroix AZ, Powles T, Osborne CK, Wolter K, Thompson JR et al (2010) Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 102:1706–1715
Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA et al (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326:852–856
Love RR, Wiebe DA, Feyzi JM, Newcomb PA, Chappell RJ (1994) Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. J Natl Cancer Inst 86:1534–1539
Malozowski S (2010) Lasofoxifene for postmenopausal women with osteoporosis. N Engl J Med 362:2227; author reply 2228–2229
Martin L, Birdsell L, Macdonald N, Reiman T, Clandinin MT et al (2013) Cancer cachexia in the age of obesity: skeletal muscle depletion is a powerful prognostic factor, independent of body mass index. J Clin Oncol 31:1539–1547
Martinborough E, Shen Y, Oeveren A, Long YO, Lau TL et al (2007) Substituted 6-(1-pyrrolidine) quinolin-2(1H)-ones as novel selective androgen receptor modulators. J Med Chem 50:5049–5052
McDonnell DP, Wardell SE (2010) The molecular mechanisms underlying the pharmacological actions of ER modulators: implications for new drug discovery in breast cancer. Curr Opin Pharmacol 10:620–628
Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS et al (2001) Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J Med Chem 44:4230–4251
Miller WL (2012) The syndrome of 17,20 lyase deficiency. J Clin Endocrinol Metab 97:59–67
Miller PD, Chines AA, Christiansen C, Hoeck HC, Kendler DL et al (2008) Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. J Bone Miner Res 23:525–535
Miner JN, Chang W, Chapman MS, Finn PD, Hong MH et al (2007) An orally active selective androgen receptor modulator is efficacious on bone, muscle, and sex function with reduced impact on prostate. Endocrinology 148:363–373
Minutolo F, Macchia M, Katzenellenbogen BS, Katzenellenbogen JA (2011) Estrogen receptor beta ligands: recent advances and biomedical applications. Med Res Rev 31:364–442
Moskovic DJ, Katz DJ, Akhavan A, Park K, Mulhall JP (2012) Clomiphene citrate is safe and effective for long-term management of hypogonadism. BJU Int 110:1524–1528
Muller JM, Isele U, Metzger E, Rempel A, Moser M et al (2000) FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J 19:359–369
Nagata N, Furuya K, Oguro N, Nishiyama D, Kawai K et al (2014) Lead evaluation of tetrahydroquinolines as nonsteroidal selective androgen receptor modulators for the treatment of osteoporosis. ChemMedChem 9:197–206
Narayanan R, Coss CC, Yepuru M, Kearbey JD, Miller DD et al (2008) Steroidal androgens and nonsteroidal, tissue-selective androgen receptor modulator, S-22, regulate androgen receptor function through distinct genomic and nongenomic signaling pathways. Mol Endocrinol 22:2448–2465
Narita D, Raica M, Suciu C, Cimpean A, Anghel A (2006) Immunohistochemical expression of androgen receptor and prostate-specific antigen in breast cancer. Folia Histochem Cytobiol 44:165–172
Nelson LR, Bulun SE (2001) Estrogen production and action. J Am Acad Dermatol 45:S116–S124
Niemeier LA, Dabbs DJ, Beriwal S, Striebel JM, Bhargava R (2010) Androgen receptor in breast cancer: expression in estrogen receptor-positive tumors and in estrogen receptor-negative tumors with apocrine differentiation. Mod Pathol 23:205–212

Nique F, Hebbe S, Triballeau N, Peixoto C, Lefrancois JM et al (2012) Identification of a 4-(hydroxymethyl)diarylhydantoin as a selective androgen receptor modulator. J Med Chem 55:8236–8247

van Oeveren A, Motamedi M, Mani NS, Marschke KB, Lopez FJ et al (2006) Discovery of 6-N, N-bis(2,2,2-trifluoroethyl)amino- 4-trifluoromethylquinolin-2(1H)-one as a novel selective androgen receptor modulator. J Med Chem 49:6143–6146

Osterberg EC, Bernie AM, Ramasamy R (2014) Risks of testosterone replacement therapy in men. Indian J Urol 30:2–7

Pedram A, Razandi M, O'Mahony F, Lubahn D, Levin ER (2010) Estrogen receptor-beta prevents cardiac fibrosis. Mol Endocrinol 24:2152–2165

Peters KM, Edwards SL, Nair SS, French JD, Bailey PJ et al (2012) Androgen receptor expression predicts breast cancer survival: the role of genetic and epigenetic events. BMC Cancer 12:132

Podo F, Buydens LM, Degani H, Hilhorst R, Klipp E et al (2010) Triple-negative breast cancer: present challenges and new perspectives. Mol Oncol 4:209–229

Prado CM, Lieffers JR, McCargar LJ, Reiman T, Sawyer MB et al (2008) Prevalence and clinical implications of sarcopenic obesity in patients with solid tumours of the respiratory and gastrointestinal tracts: a population-based study. Lancet Oncol 9:629–635

Ryan WG, Wolter J, Bagdade JD (1991) Apparent beneficial effects of tamoxifen on bone mineral content in patients with breast cancer: preliminary study. Osteoporos Int 2:39–41

Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE et al (2001) Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci U S A 98:4904–4909

Sasano H, Miki Y, Nagasaki S, Suzuki T (2009) In situ estrogen production and its regulation in human breast carcinoma: from endocrinology to intracrinology. Pathol Int 59:777–789

Schlienger N, Lund BW, Pawlas J, Badalassi F, Bertozzi F et al (2009) Synthesis, structure-activity relationships, and characterization of novel nonsteroidal and selective androgen receptor modulators. J Med Chem 52:7186–7191

Schmidt A, Harada S, Kimmel DB, Bai C, Chen F et al (2009) Identification of anabolic selective androgen receptor modulators with reduced activities in reproductive tissues and sebaceous glands. J Biol Chem 284:36367–36376

Sharifi N, Auchus RJ (2012) Steroid biosynthesis and prostate cancer. Steroids 77:719–726

Simpson ER, Zhao Y, Agarwal VR, Michael MD, Bulun SE et al (1997) Aromatase expression in health and disease. Recent Prog Horm Res 52:185–213 discussion 213–184

Smith CL, O'Malley BW (2004) Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 25:45–71

Smith MR, Malkowicz SB, Brawer MK, Hancock ML, Morton RA et al (2011) Toremifene decreases vertebral fractures in men younger than 80 years receiving androgen deprivation therapy for prostate cancer. J Urol 186:2239–2244

Tan RS, Teoh SH (2013) Testosterone use in women: how safe is it? Curr Drug Saf 8:120–127

Tan BH, Birdsell LA, Martin L, Baracos VE, Fearon KC (2009) Sarcopenia in an overweight or obese patient is an adverse prognostic factor in pancreatic cancer. Clin Cancer Res 15:6973–6979

Tsoli M, Robertson G (2013) Cancer cachexia: malignant inflammation, tumorkines, and metabolic mayhem. Trends Endocrinol Metab 24:174–183

Vajda EG, Lopez FJ, Rix P, Hill R, Chen Y et al (2009) Pharmacokinetics and pharmacodynamics of LGD-3303 [9-chloro-2-ethyl-1-methyl-3-(2,2,2-trifluoroethyl)-3H-pyrrolo-[3,2-f]quinolin-7(6H)-one], an orally available nonsteroidal-selective androgen receptor modulator. J Pharmacol Exp Ther 328:663–670

Vigen R, O'Donnell CI, Baron AE, Grunwald GK, Maddox TM et al (2013) Association of testosterone therapy with mortality, myocardial infarction, and stroke in men with low testosterone levels. JAMA 310:1829–1836

Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS et al (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 295:2727–2741

von Haehling S, Anker SD (2012) Cachexia as major underestimated unmet medical need: facts and numbers. Int J Cardiol 161:121–123

Wang F, Liu XQ, Li H, Liang KN, Miner JN et al (2006) Structure of the ligand-binding domain (LBD) of human androgen receptor in complex with a selective modulator LGD2226. Acta Crystallogr Sect F Struct Biol Cryst Commun 62:1067–1071

Wilson DE, Kaidbey K, Boike SC, Jorkasky DK (1998) Use of topical corticosteroid pretreatment to reduce the incidence and severity of skin reactions associated with testosterone transdermal therapy. Clin Ther 20:299–306

Wood CE, Kaplan JR, Fontenot MB, Williams JK, Cline JM (2010) Endometrial profile of tamoxifen and low-dose estradiol combination therapy. Clin Cancer Res 16:946–956

Wu YL, Yang X, Ren Z, McDonnell DP, Norris JD et al (2005) Structural basis for an unexpected mode of SERM-mediated ER antagonism. Mol Cell 18:413–424

Xie W, Evans RM (2001) Orphan nuclear receptors: the exotics of xenobiotics. J Biol Chem 276:37739–37742

Yin D, Gao W, Kearbey JD, Xu H, Chung K et al (2003) Pharmacodynamics of selective androgen receptor modulators. J Pharmacol Exp Ther 304:1334–1340

Zhang X, Lanter JC, Sui Z (2009) Recent advances in the development of selective androgen receptor modulators. Expert Opin Ther Pat 19:1239–1258

Zhou X (2010) Roles of androgen receptor in male and female reproduction: lessons from global and cell-specific androgen receptor knockout (ARKO) mice. J Androl 31:235–243

Index

A
Aagaard, M.M. 94
Abdel-Hafiz, H. 29
Ackermann, D. 25
Ackers, G.K. 78
Activation function 2 (AF-2) 19, 138, 143, 144
Adachi, S. 84
Adair, G.B.A.F. 78
Agonists 3, 100, 139, 179
  binding 2, 3, 123
Ahmad, N. 41
Alberts, B.M. 76
Aldosterone 19, 24, 27–30
  receptor 17
Alen, P. 60
Allan, G.F. 73, 75
Allosteric model 77, 78, 84
Allosteric regulation 3, 5, 7, 11, 51, 52
  mechanisms of 7
  of DNA 7
Almlof, T. 49
Alnemri, E.S. 111
Amino-terminal domain (NTD) 60, 69, 157
  of TBL1X 159, 160
Amsterdam, A. 78, 82
Androgen receptor (AR) 66, 69, 110, 205
  structural constraints on 137–139
Antoniewski, C. 94
Aoyagi, S. 120
Aranda, A. 83
Arbeitman, M.N. 107
Archer, T.K. 120
Ariyoshi, M. 167
Arnold, A.L. 190
Arrar, M. 161
Arriza, J.L. 24
Ashok, M. 107, 108
Askew, E.B. 137, 141, 143
Auboeuf, D. 26
Auzou, G. 23
Axerio-Cilies, P. 192

B
Bagchi, M.K. 76
Bai, S. 137, 138, 141–144, 147
Bain, D.L. 74, 76, 82
Baker, K.D. 103
Banuelos, S. 110
Barcroft, J. 78
Barnes, P.J. 41
Baskakov, I.V. 48, 74, 75
Baudrand, R. 30
Baumann, A. 108
Baumann, H. 47
Beato, M. 42
Beck, I.M. 41, 43, 49
Becker, P.B. 76
Beekman, J.M. 73
Benhamou, B. 20
Bennett, M.J. 139
Berglund, H. 74
Bernardo, T.J. 108, 109
Bielska, K. 105, 106
Billas, I.M. 99–101, 120
Billas, I.M.L. 123, 126
Binart, N. 21
Binding function 3 (BF3) 181
Blackford, J.A., Jr. 82, 83
Blasi, E.R. 30
Bledsoe, R.K. 19–23, 43, 46, 48
Bohl, C.E. 9
Bolen, D.W. 74
Bolton, E.C. 65
Bookout, A.L. 27
Borden, S.A. 30

I. J. McEwan, R. Kumar (eds.), *Nuclear Receptors: From Structure to the Clinic*,
DOI 10.1007/978-3-319-18729-7

Bourguet, W. 2, 73, 119, 123
Boyd, S. 205
Brelivet, Y. 120, 124
Brodie, J. 25, 51, 68, 76
Brown, M. 76
Browning, C. 99–101
Bruner, K.L. 21
Brzozowski, A.M. 2, 23, 48, 73
Buljan, M. 77
Bulynko, Y.A. 26, 120
Burchardt, T. 147
Busillo, J.M. 17

C
Callewaert, L. 60, 67, 68
Cao, Y. 77
Carlstedt-Duke, J. 1
Carmichael, J.A. 99, 101
Carson, M.W. 48
Castrate resistant prostate cancer (CRPC) 180, 208
Cato, A.C. 60
Cato, A.C.B. 25
Chakraborty, S. 196
Chambon, P. 4, 23
Chandra, V. 1, 2, 5–8, 50–52, 120
Chang, C.Y. 141
Charles, J.P. 108
Chatterjee, V.K. 8
Chaumot, A. 101
Chd64 109, 110
Chellappan, S.P. 145
Chen, J.D. 28
Chen, W. 49
Cherbas, L. 94
Choi, J.H. 130
Choong, C.S. 138, 141, 142
Chromatin 26, 60, 61, 155, 160, 168, 179
  in vivo 64
Cidlowski, J.A. 17, 41, 44, 45, 77, 84
Claessens, F. 4, 5, 59–62, 65, 66, 68
Clayton, G.M. 101
Cleutjens, K.B. 65
Cleve, A. 9
Clinckemalie, L. 67
Cluning, C.I. 22
Conformational dynamics 43, 47, 48, 51, 84
  importance of 43
Conway-Campbell, B.L. 29
Copik, A.J. 75, 76
Co-regulatory proteins 11, 81, 196
Corepressor complexes 120, 155, 156, 168
  formation of 64
Cortisol 17, 19, 20, 22, 206
Couture, J.-F. 160
Cronet, P. 166
Cryo-electron microscopy 51

D
Dahlman-Wright, K. 48, 74
Dai, S.Y. 51
Daily, M.D. 78
Darimont, B.D. 3
De Bellis, A. 140
De Bosscher, K. 43, 48
De Bruyn, R. 61
de Lange, P. 77
De Leon, J.T. 194
De Plaen, E. 142
Dehm, S.M. 146
Dejean, A. 29
Dela Cruz, F.E. 77, 102
DeLano, W.L. 96, 100
Demarest, S.J. 50
Denayer, S. 61, 63, 64
Devarakonda, S. 51, 95, 96
Diamanti-Kandarakis, E. 83
Dietz, J.D. 30
Direct repeat 5, 51, 65
  classical 122
  motifs 63
DNA binding domain (DBD) 3, 42, 97, 119
Dobryszycki, P. 97, 98
Domain coupling 75
Dooley, R. 30
Dougherty, J.J. 84
Doyle, J.M. 143, 148
Doyon, Y. 155
Dubbink, H.J. 3
Dubois, V. 59
Dubrovsky, E.B. 108, 109
Duff, J. 8
Dummitt, B. 99
Dunker, A.K. 110, 138, 148
Dutko-Gwóźdź, J. 98
Dyson, H.J. 102, 103, 138, 141
Dziedzic-Letka, A. 102, 103

E
Ecdysteroid receptor (EcR) 93, 94, 105
Ecdysteroid response elements (EcRE) 93
Egea, P.F. 3, 123
Eisenberg, D. 138
Eriksson, M.A. 47
Estébanez-Perpiñá, E. 10, 196
Estrogen receptor α (ERα) 23, 76
Estrogen receptor β (ERβ) 59
Evans, R.M. 1, 28, 42

F
Fabian, J. 108
Fagart, J. 19, 20, 22, 23, 28, 30
Fang, X. 99
Faresse, N. 21
Fattaey, A.R. 145
Fattori, J. 129
Feldman, D. 17, 30
Fernandez, E.J. 5
Ferreon, A.C.M. 78
Fiebeler, A. 25
Finnin, M.S. 156
Fischer, K. 19, 50, 74
Fisk, G.J. 94
FK506-binding protein (FKBP39) 109–111
Ford, J. 49
Forman, B.M. 76
Frankfurt, O. 41
Freedman, L.P. 67
French, F.S. 137, 138
Frey, F.J. 43
Fu, M. 84
Fuller, P. 19
Fuller, P.J. 18, 19, 24
Funder, J.W. 17
Fuse, H. 19

G
Gaillard, E. 120
Galat, A. 110
Galigniana, M.D. 21
Gallastegui, N. 2, 3
Gallo, L.I. 22
Gampe, R.T. 123
Gao, J. 104
Garcia-Carpizo, V. 83
Garside, H. 76
Garza, A.S. 49
Gee, A.C. 74
Geerling, J.C. 17
Geiss-Friedlander, R. 105
Geller, D.S. 22, 23, 30
Geller, J. 146
Gelmann, E.P. 141
Gene regulation 3
  physiological 111
  receptor-selective 4
  target-specific 45
Gerber, A.N. 76
Geserick, C. 25, 69, 76
Gewirth, D.T. 4, 5
Gilbert, L.I. 105
Gill, S. 79
Gimona, M. 110
Glass, C.K. 74, 75, 120
Glucocorticoid receptor (GR) 1–3, 17, 41, 43, 74
Godlewski, J. 108
Gomez-Sanchez, E.P. 17
Govindan, M.V. 19
Grad, I. 96
Gray, J.J. 78
Greb-Markiewicz, B. 109
Green, S. 4, 23
Greene, G.L. 76
Greenfield, N. 76
Gregory, C.W. 146
Groeneweg, F.L. 30
Grontved, L. 64
Gros, R. 30
Grosse, A. 64
Grossman, C. 21, 24, 30
Guo, C. 30
Guo, E. 108
Guo, Z. 138
Gurnell, M. 8
Gustafsson, J.A. 1

H
Haelens, A. 64
Haendler, B. 9
Hake, S.B. 83
Han, S.J. 82
Hard, T. 4, 5, 47
Harris, S.E. 141
Hartwig, J.H. 110
Hasui, T. 30
He, B. 2, 3, 8, 23, 137–139, 141
He, Y. 43, 48
Heemers, H.V. 141
Heery, D.M. 3, 25
Helsen, C. 51, 63, 68
Henriksson, J. 49
Henry, E.R. 78
Heterodimerization 5–7, 101, 196
Heyduk, E. 97, 99
Heyduk, T. 99
Hill, A.V. 78
Hilser, V.J. 78, 80, 81
Himukai, R. 111
Hiort, O. 140
Hirata, A. 30
Hirschberg, D. 28
Hittelman, A.B. 51
Ho, K.C. 137
Hodges, C.V. 205
Hoffmann, R. 147
Hogness, D.S. 107

Hollenberg, S.M. 48
Hong, K.W. 138
Horlein, A.J. 28
Housley, P.R. 84
Hsieh, J.C. 127
Hsu, C.L. 138
Hsu, P.Y. 83
Hu, X. 102, 106
Hua, S. 122
Huang, P. 3
Huang, W. 137
Hudson, W.H. 4, 74
Huet, T. 123
Huggins, C.S.R. 205
Hultman, M.L. 27
Hur, E. 3
Hurtado, A. 60
Huyet, J. 19, 21

I
Iakoucheva, L.M. 105
Igarashi, T.M. 145
Ikeda, M. 75, 76, 82
Ikonen, T. 60
Inaba, H. 41
Iniguez-Lluhi, J.A. 29, 67
Inositol phosphate 161–163, 169
Intrinsic disordered structure
  of NTD 7
Intrinsically disordered proteins (IDP) 48, 73, 102
Irvine, R.A. 60
Ivarie, R.D. 75
Iwema, T. 99, 101

J
Jakób, M. 95–98, 121
James, S.R. 147
Jenkins, B.D. 83
JH (Juvenile hormone) 101, 107
  receptor 107–109
Jia, L. 65
Jin, L. 165
Jindra, M. 107–109
John, S. 64
Johnson, B.H. 41
Jonat, C. 25
Jones, D. 107
Jones, G. 101, 107
Jordan, V.C. 78, 82
Juurlink, D.N. 30

K
Kallenberger, B.C. 8, 73
Kangaspeska, S. 83
Kapitskaya, M. 103
Karpf, A.R. 142, 144, 147
Karst, H.I. 30
Kastner, P. 77
Katzenellenbogen, J.A. 216
Keightley, M.C. 20
Kemppainen, J.A. 139
Kerkhofs, S. 63
Khan, S.H. 49, 50, 75
King, W.J. 76
Kino, T. 29
Klokk, T.I. 24
Knoll, E. 99
Knutti, D. 83
Kolb-Kokocinski, A. 142
Kolkhof, P. 30
Kolla, V. 25
Konopova, B. 108
Koshland, D.E., Jr. 78
Kotaja, N. 60
Kouzarides, T. 155
Kozłowska, M. 110
Kramer, F.R. 99
Krasowski, M.D. 73, 74, 77, 102
Krusiński, T. 99
Kumar, R. 7, 11, 42, 43, 45, 46, 48–51, 74–76, 82

L
La Spada, A.R. 138
Labrie, C. 146
Lack, N.A. 10, 194
Lagarde, W.H. 141
Lalevee, S. 28
Lane, D. 97
Langley, E. 23, 139
Lanzino, M. 64, 65
Laudet, V.G.H. 94, 106
Laurent, M. 59
Lavery, D.N. 19, 74, 75, 137
Le Drean, Y. 84
Le Maire, A. 123, 165
Lefstin, J.A. 49, 74, 75
Lehmann, M. 94
Leon, C.G. 147
Li, J. 75
Li, J.Q. 108, 109
Li, Y. 19, 20, 23, 27, 107, 109, 110
Liden, J. 25

Ligand binding domain (LBD) 2, 42, 119, 141, 179, 180
Ligand binding pocket (LBP) 2, 3, 9, 10, 52, 53, 215, 216, 221
Lim, J. 139
Lim-Tio, S.S. 20
Litwack, G. 25
Liu, J. 73
Liu, P.C. 107, 110
Liu, Q. 138, 142, 143
Liu, W. 25
Liu, X. 146
Liu, Y. 108
Locke, J.A. 146
Lockless, S.W. 78
Loewy, A.D. 17
Lombes, M. 24
Lonard, D.M. 155
Lou, X. 1, 2, 5, 6, 120
Loven, M.A. 25, 74, 76
Lu, N.Z. 44, 45, 77
Lubahn, D.B. 69, 137, 140
Luisi, B.F. 24, 46, 74, 121
Lund, S.D. 137
LxxLL motifs 7, 24, 26, 138, 193

M
Ma, H. 60
Madauss, K.P. 165
Mader, S. 122
Malaney, P. 104
Malartre, M. 165
Maletta, M. 96, 120
Marmorstein, R. 146
Martinez, E. 75
Martinez, E.D. 21
Massaad, C. 22
McDonnell, D.P. 9
McEneaney, V. 29
McEwan, I.J. 7, 8, 11, 19, 25, 48–51, 60, 68, 74–76, 93, 102, 107, 137
McGough, A. 110
McInerney, E.M. 26
McKenna, N.J. 155
McNally, J.G. 51
Meijsing, S.H. 4, 5, 25, 46, 47, 74, 82, 121
Melanoma antigen-A11 (MAGE-A11) 138, 139, 141, 142, 144, 147, 148
Melchior, F. 105
Merkle, D. 147
Merry, D.E. 138
Methoprene-tolerant protein (Met) 107, 108
Metivier, R. 23, 155
Meyer, M.E. 76, 77
Meyers, M.J. 30
Miesfeld, R. 48
Millard, C.J. 162
Miner, J.N. 43
Mineralocorticoid receptors (MR) 4, 17, 182
  structure 18, 19
Minges, J.T. 142, 143
Mital, R. 110
Miura, K. 107, 108
Mohler, J.L. 146
Monod, J. 78
Montgomery, R.B. 146
Moore, A.W. 108
Mostaghel, E.A. 146
Motlagh, H.N. 78
Mouillet, J.F. 77, 102
Moutier, E. 122
Murai-Takeda, A. 28

N
N/C-terminal interactions 4, 18, 21, 24, 81, 96
Nagaich, A.K. 51
Nagy, L. 3, 120
Nahoum, V. 73, 123
Nair, R.R. 110
Nakshatri, H. 28
Narayanan, R. 9
Nettles, K.W. 10
Nicolai, M. 105
Nie, Z. 83
Niedziela-Majka, A. 94–97
NMR spectroscopy 10
Nocula-Ługowska, M. 74, 102
Nolte, R.T. 166
Nordeen, S.K. 22
Norman, A.W. 83
Nuclear receptor corepressor (NCoR) 27, 28, 156, 164
Nuclear receptor superfamily 1, 19, 26, 93
Nuclear receptors 2, 6, 156, 164–166, 168, 179
  antagonist-bound 157
  non-steroid 4

O
O'Malley, B.W. 26, 120, 155
Oakley, R.H. 41, 45
Oberoi, J. 3
Odermatt, A. 17
Orlov, I. 51, 120, 125
Orłowski, M. 94–97
Orr, C.R. 138

Ostrowski, J. 9
Osz, J. 51, 120, 122, 129
Ou, X.-M. 25
Ożyhar, A. 94

P
Pakuła, S. 98
Parker, M.G. 61
Parthun, M.R. 155
Pascual, A. 83
Pascual-Le Tallec, L. 19, 27
Paul, A. 22
Pavlin, M.R. 7
Payvar, F. 61
Pearce, D. 25, 29
Pelham, H.R. 94
Perera, S.C. 94
Perlmann, T. 122
Perutz, M.F. 78
Petit, C.M. 78
Petoukhov, M.V. 121
Petz, L.N. 97
Phelan, C.A. 165
Phillips, C. 183
Pieprzyk, J. 102–104
Pike, A.C. 3, 9
Pike, J.W. 84
Pippal, J.B. 19, 23, 24
Pitt, B. 17, 30, 31
Pogenberg, V. 123
Poletti, A. 138
Pongs, O. 94
Potthoff, S.J. 97
Poukka, H. 29, 67, 84
Pratt, W.B. 21, 43, 84
Prefontaine, K.E. 25
Prendergast, P. 97
Prilusky, J. 47
Pui, C.H. 41
Pursley, S. 108
Putcha, B.D. 5
Putcha, B.-D.K. 123

Q
Qi, W. 64
Qin, S. 155
Quigley, C.A. 23, 139, 140

R
Rafestin-Oblin, M.E. 21, 22
Rastinejad, F. 2, 4, 5, 95, 96, 121, 127
Rauch, P. 105
Ravindranathan, P. 192
Ray, A. 25
Record, M.T., Jr. 74
Reichardt, H.M. 42
Reichheld, S.E. 78
Reid, J. 50, 67, 68, 75
Renaud, J.P. 2, 73
Response element 129
  DNA 1, 3–5, 125
Retinoid X Receptor (RXR) 4–6, 124, 125, 127, 128
Richer, J.K. 145
Rickard, A.J. 17, 30
Riddiford, L.M. 107, 109
Riddihough, G. 94
Roche, P.J. 61, 62
Rochel, N. 5–7, 51, 120, 125, 128, 130
Roemer, S.C. 5
Rogerson, F.M. 17, 19, 20, 22–24, 27
Rogner, U.C. 142
Ronacher, K. 76
Rosen, J. 43
Rosen, S.T. 41
Rosenfeld, M.G. 120
Ross, M.T. 142
Rupprecht, R. 20
Russcher, H. 77
Ruthenburg, A.J. 160
Rymarczyk, G. 104

S
Sack, J.S. 137
Sahu, B. 64, 66
Sakai, D.D. 75
Sang, M. 143
Santos, G.M. 155
Sato, A. 17
Sato, Y. 123
Savory, J.G.A. 19, 25
Schaufele, F. 24, 139
Schauwaers, K. 63
Scheller, A. 67
Schiller, B.J. 47
Schmid, F.X. 111
Schmidt, A. 139
Schoch, G.A. 48, 165
Schoenmakers, E. 60, 62, 63
Schrader, W.T. 77
Schutz, G. 42
Schwabe, J.W.R. 2–5, 74, 121, 167
Seeler, J.-S. 29
Selective ARE 62, 63, 68
Seliga, J. 105, 106
Shaffer, P.L. 4, 5, 63, 127

Shang, Y. 76
Sharp, P.A. 107
Shavit, L. 30
Shemshedini, L. 107
Shulemovich, K. 97
Simental, J.A. 137
Simons, S.S. Jr. 49
Sleator, N.M. 84
Smith, F.R. 78
SMRT 27, 156, 157, 159, 161, 167
  peptide 167
So, A.Y. 59
Song, Q. 105
SPecificity affecting AR Knock In (SPARKI) model 63, 64
Spolar, R.S. 74
Starr, D.B. 74, 75
Stenoien, D.L. 51, 138
Stow, L.R. 24
Su, S. 142, 145
Suel, G.M. 78
Suino, K. 123
Sumoylation 24, 27, 29, 60, 169
Sun, X. 105
Sutherland, J.D. 94, 98, 103
Svensson, S. 123
Swanson, W.J. 142
Szamborska-Gbur, A. 95
Szapary, D. 83

T
Tagami, T. 155
Takacs, M. 120, 129
Takayama, K. 65
Talbot, W.S. 77, 102
Tan, J. 141
Tan, J.-A. 27
Tanenbaum, D.M. 2
Tanner, T.M. 60, 64
Tao, Y.G. 48
Taslim, C. 65
Taunton, J. 156
Tay, S.K. 142
Tetel, M.J. 23, 145
Tewari, A.K. 61
Thompson, E.B. 41–43, 45, 46, 49, 74, 76, 78, 80–82
Thompson, J.D. 23, 104
Thummel, C.S. 94
Tilley, W.D. 68
Tindall, D.J. 141
Tirard, M. 27, 29
Titus, M.A. 146
Toft, D.O. 21, 43
Tompa, P. 73, 102
Tontonoz, P. 77
Transcriptional regulation 49, 77, 105
Trapp, T. 25
Truman, J.W. 82
Tsai, M.J. 60
Tsuji, M. 19
Turner, B.M. 156
Twiddy, A.L. 147
Tyagi, S. 99

U
Ultraspiracle (Usp) 93, 95, 120, 126
Umesono, K. 62
Uversky, V.N. 74, 102, 105, 110

V
Vacquier, V.D. 142
Van Duyne, G.D. 111
van Royen, M.E. 139
Veesler, D. 121
Velasco, L.F.R. 125
Veldscholte, J. 141
Verrijdt, G. 60, 62, 63
Viengchareun, S. 28, 29
Vivat, V. 21
Vlassi, M. 19

W
Wagner, R.L. 73
Wahli, W. 75
Wallace, A.D. 84
Walters, K.A. 82
Walther, R.F. 29
Wang, D. 50
Wang, F. 9
Wang, L. 165
Wang, Q. 27, 61, 63, 65, 67
Wang, S.-F. 94, 103, 105, 108
Wang, Z. 155
Warnmark, A. 49, 50, 75
Warriar, N. 19
Watanabe, T. 105
Watkins, R.E. 166
Watson, L.C. 4, 47, 51, 52
Watson, P.A. 146
Watson, P.J. 161
Watters, A.L. 74
Webb, P. 164
Webster, J.C. 84
Welshons, W.V. 76

Wilson, E.M. 137–139, 143
Wilson, T.G. 107, 108, 110
Wochnik, G.M. 22
Wolf, D.M. 78, 82
Wong, C.I. 143
Wood, J.R. 74, 76, 82
Wrange, O. 1
Wright, P.E. 102, 103, 138, 141
Wyman, J. 79
Wysocka, J. 160

X
Xie, H. 104
X-ray crystallography 10, 73, 119, 181, 194, 197, 199
Xu, D. 104
Xu, H.E. 130, 165
Xu, R.X. 123
Xu, Y. 101

Y
Yamamoto, K.R. 25, 49, 76
Yang, J. 18, 26, 27
Yang, Z. 110
Yao, T.P. 93
Yi, P. 11
Yokota, K. 27, 29
Young, M.J. 26, 30

Z
Zannad, F. 17, 30
Zegarra-Moro, O.L. 146
Zhang, H. 165
Zhang, J. 5, 7, 22, 23, 51, 125
Zhang, M. 64, 65
Zhang, Z. 109
Zhao, Q. 121
Zhou, Z.X. 138, 139
Zoglowek, A. 94, 98, 102