



Publicly Accessible Penn Dissertations

Spring 5-17-2010

Molecular and Structural Insights into Nuclear Hormone Receptor Repression Mediated by the Corepressor NCOR

Caroline A. Murphy

University of Pennsylvania, camurphy@mail.med.upenn.edu

Follow this and additional works at: <http://repository.upenn.edu/edissertations>

 Part of the [Pharmacology Commons](#)

Recommended Citation

Murphy, Caroline A., "Molecular and Structural Insights into Nuclear Hormone Receptor Repression Mediated by the Corepressor NCOR" (2010). *Publicly Accessible Penn Dissertations*. 150.
<http://repository.upenn.edu/edissertations/150>

This paper is posted at ScholarlyCommons. <http://repository.upenn.edu/edissertations/150>
For more information, please contact libraryrepository@pobox.upenn.edu.

Molecular and Structural Insights into Nuclear Hormone Receptor Repression Mediated by the Corepressor NCOR

Abstract

Nuclear hormone receptors comprise a large family of ligand-sensitive transcription factors that can directly bind and regulate target genes to affect various physiological processes including development, differentiation, circadian rhythm and metabolism. Classically, activation of transcription by nuclear receptors (NRs) is due to a ligand-induced switch from corepressor- to coactivator-bound states. Highly analogous corepressors including NCoR and SMRT facilitate repression by NR via recruitment and activation of the histone deacetylase HDAC3. Liver X Receptor (LXR) is an NR that functions to regulate diverse physiological processes including cholesterol metabolism, lipid homeostasis, immunity and inflammation. Selective modulators of LXR to specifically target pathways for peripheral cholesterol efflux were developed and observed to function as partial agonists of LXR. We determined that the selective partial agonism of LXR by these ligands was indeed related to differential recruitment of the corepressor NCoR. Secondly, to understand the structural basis for physiological repression of NR by NCoR we co-crystallized a small peptide comprising a region of the interaction domain of NCoR with the ligand-binding domain of Rev-erba. This revealed that the relative structural requirements for the previously reported antagonized PPAR α -bound SMRT were distinct from that of the Rev-erba:NCoR complex. Altogether, these studies provide novel molecular insight into the function of NCoR in regulating transcription by nuclear hormone receptors.

Degree Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

First Advisor

Mitchell A. Lazar, M.D., Ph.D.

Keywords

Transcription, Nuclear receptors, Circadian biology

Subject Categories

Pharmacology

MOLECULAR AND STRUCTURAL INSIGHTS INTO NUCLEAR HORMONE
RECEPTOR REPRESSION MEDIATED BY THE COREPRESSOR NCOR

CAROLINE A. MURPHY

A DISSERTATION

in

PHARMACOLOGICAL SCIENCES

Presented to the faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
2010

Supervisor of Dissertation

Signature_____

Mitchell A. Lazar, M.D., Ph.D

Graduate Group Chairperson

Signature_____

Vladimir R. Muzykantov, M.D., Ph.D.

Dissertation Committee:

Klaus H. Kaestner, Ph.D.

John B. Hogenesch, Ph.D.

Gerd A. Blobel, M.D., Ph.D.

Edward E. Morrisey, Ph.D.

DEDICATION

This thesis is dedicated to my best friend and husband Tim. He has been a tremendous support throughout graduate school and I am indebted to him for his love and understanding over these past several years. I also dedicate this work to my parents, Tom and Sue Murphy, who have always understood my passion for science and learning and for their immense love.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis mentor Mitch Lazar for his tremendous support over the years. He provided much needed guidance and knowledge throughout this process and I cannot think of someone I could more hope to emulate over my career. He is an amazing friend, colleague, mentor and one of the most talented scientists I will likely ever come across. His amazing passion for science and his position as a thought-leader in our field has been truly inspiring and something I will carry with me and try to mirror in my own career. I am forever indebted to him for his wonderful mentorship at Penn. I would also like to thank my thesis committee, in particular, Klaus Kaestner who was my chair for their intuitive advice and discussions during the course of this work. The committee including Ed Morrissey, Gerd Blobel and John Hogenesch was invaluable for the successful completion of this dissertation.

I also want to thank the Lazar lab for the amazingly friendly and collegial environment. I am particularly grateful to Joe Weaver, Lei Yin, Nan Wu and my bay-mate Takuya Tomaru for their scientific advice and friendship.

Throughout my career in science I have made some particularly amazing friends who happen to be scientists too. I especially cherish the friendship of Lisa Vuchak and Dan Leung, my 2004 pharmacology classmates, as well as other wonderful Penn friends including Dr. Lau, Amy Quinn, Jason Drury, Erin Griner, Lindsay McKenna, Nuria Bramswig, and Allison Leshner. I also want to thank my wonderful friends at SIAL, Jon Stephan, Andrea Spencer, Natalie Greene, Rebecca Pashia and Kyle Brueggeman.

Beyond Penn, I want to thank my twin sister and best friend, Maureen Chiodo, Tim Chiodo, Eileen Murphy, my grandma Margaret Murphy, Bridget Murphy, Gwen Murphy, Ryan Murphy and Kathleen Murphy. I also want to thank my in-laws Mike and Donna Phelan. In addition, my cherished friends and fellow bulldogs from undergrad Cathy Chirco, Megan Keyes, Krista LaRocca, Michelle Twellman, Emily Leas, Katie Nelson and Amy Huntbach.

ABSTRACT

**MOLECULAR AND STRUCTURAL INSIGHTS INTO NUCLEAR
HORMONE RECEPTOR REPRESSION MEDIATED BY THE
COREPRESSOR NCoR**

Caroline A. Murphy

Advisor:

Mitchell A. Lazar M.D, Ph.D.

Nuclear hormone receptors comprise a large family of ligand-sensitive transcription factors that can directly bind and regulate target genes to affect various physiological processes including development, differentiation, circadian rhythm and metabolism. Classically, activation of transcription by nuclear receptors (NRs) is due to a ligand-induced switch from corepressor- to coactivator-bound states. Highly analogous corepressors including NCoR and SMRT facilitate repression by NR via recruitment and activation of the histone deacetylase HDAC3. Liver X Receptor (LXR) is an NR that functions to regulate diverse physiological processes including cholesterol metabolism, lipid homeostasis, immunity and inflammation. Selective modulators of LXR to specifically target pathways for peripheral cholesterol efflux were developed and observed to function as partial agonists of LXR. We determined that the selective partial agonism of LXR by these ligands was indeed related to differential recruitment of the corepressor NCoR. Secondly, to understand the structural basis for physiological

repression of NR by NCoR we co-crystallized a small peptide comprising a region of the interaction domain of NCoR with the ligand-binding domain of Rev-erb α . This revealed that the relative structural requirements for the previously reported antagonized PPAR α -bound SMRT were distinct from that of the Rev-erb α :NCoR complex. Altogether, these studies provide novel molecular insight into the function of NCoR in regulating transcription by nuclear hormone receptors.

CONTENTS

DEDICATION.....	ii
ACKNOWLEDGMENTS.....	iii
ABSTRACT.....	iv
CONTENTS.....	vi
LIST OF TABLES AND FIGURES.....	viii
ABBREVIATIONS USED.....	ix
CHAPTER ONE: GENERAL INTRODUCTION.....	1
1. Nuclear Hormone Receptors	
1.1 Classes of Nuclear Hormone Receptors	
1.2 The Modular Structure of Nuclear Hormone Receptors	
1.3a DNA-binding domain and NHR Target Sequence Recognition	
1.3b Ligand-Binding Domain	
1.3c A/B Domain and Ligand-Independent Activation Function-1 Activity	
1.3d Hinge/D Domain	
2. Nuclear Hormone Receptor Mechanism of Action	
2.1 Allosteric Effects of DNA Binding	
2.2 Ligand Mediated Regulation of LBD Activity	
2.3 Coactivators	
2.4 Corepressors	
3. Physiological Function of LXR	

4. Physiological Function of Rev-erb

**CHAPTER TWO: SELECTIVE PARTIAL AGONISM OF LIVER X RECEPTOR
ALPHA IS RELATED TO DIFFERENTIAL COREPRESSOR
RECRUITMENT.....30**

- 1. Introduction**
- 2. Methods**
- 3. Results**
- 4. Discussion**
- 5. Acknowledgments**

**CHAPTER THREE: STRUCTURAL ANALYSIS OF REV-ERB α REPRESSION
BY THE NUCLEAR COREPRESSOR NCoR.....56**

- 1. Introduction**
- 2. Methods**
- 3. Results**
- 4. Discussion**
- 5. Acknowledgments**

CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS.....82

REFERENCES.....95

LIST OF FIGURES AND TABLES

- Figure 1.1** General schematic of nuclear receptor structure
- Figure 1.2** General schematic of corepressor modular domains
- Figure 1.3** Mechanism of corepressor exchange
- Figure 1.4** LBD Helix 12 Repositioning
- Figure 2.1** Transactivation by LXR Ligands Disproportionate to Coactivator Recruitment
- Table 2.1** LXR Ligand Properties
- Figure 2.2** LXR Ligands Differentially Dismiss Corepressor CoRNR Peptide *in Vitro*
- Figure 2.3** Differential Dismissal of Corepressor from LXR on an LXR α -Responsive Reporter Gene
- Figure 2.4** Modulation of NCoR Levels Differentially Influences Ligand-Dependent Transactivation of LXR α
- Figure 2.5** Differential Activation of Full-Length LXR
- Figure 2.6** Endogenous Gene Regulation by LXR Ligands in Hepatoma Cells
- Figure 2.7** LXR Ligands Differentially Recruit NCoR to Endogenous Target Genes
- Figure 2.8** NCoR Depletion Induces Basal Activation of LXR Target Genes and Differentially Potentiates Ligand-Dependent Gene Activation
- Figure 3.1** Model of two-turn vs. three-turn for interaction domains of NCoR and SMRT
- Figure 3.2** Co-crystallization of Rev-erb α Δ 323-423LBD and NCoR Interaction Domain 1 (2040-2065)
- Figure 3.3** Molecular determinants of Rev-erb α /NCoR Interaction
- Figure 3.4** Key Hydrophobic Residues of Rev-erb α which mediate NCoR binding are required for Rev-erb α repression function
- Figure 3.5** NCoR binds Rev-erb α differently than antagonized PPAR α binds SMRT
- Figure 3.6** Rev-erb α repression requires the IxxII CoRNR box motif of NCoR
- Figure 3.7** Nuclear hormone receptors utilize different modes of corepressor binding

ABBREVIATIONS USED

TR	thyroid hormone receptor
LBD	ligand binding domain
DBD	DNA binding domain
RXR	retinoid X receptor
LXR	liver X receptor
FXR	farnesoid X receptor
RAR	retinoic acid receptor
ER	estrogen receptor
VDR	vitamin D receptor
PR	progesterone receptor
PPAR	peroxisome proliferator-activated receptor
GRIP-1	glucocorticoid receptor interacting protein 1
NCoR	nuclear receptor corepressor 1
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
HRE	hormone response element
DR	direct repeat
DR-4	direct repeats spaced by 4 base pairs
DR-2	direct repeats spaced by 2 base pairs

CHAPTER ONE

GENERAL INTRODUCTION

1. Nuclear Hormone Receptors (NHRs)

Nuclear hormone receptors represent a diverse and evolutionarily conserved class of transcription factors that serve to sense both the cellular and external environment by association with cognate ligands which include steroids, lipids and fatty acids, metabolites, and signaling molecules such as heme (Gronemeyer et al., 2004). Physiologically, these receptors play integral roles in the regulation of growth, differentiation, endocrine disorders and metabolism, and circadian rhythm (Mangelsdorf et al., 1995; Yang et al., 2006). Due to their ability to directly regulate these pathways via direct DNA binding of their target genes as well as the nature of their small, hydrophobic ligands, NRs constitute extremely attractive therapeutic targets. Among the 48 human NR, their respective pharmacological agents including tamoxifen (estrogen receptor), dexamethasone (glucocorticoid receptor) and thiazolidiones, or TZDs (PPAR γ) constitute some of the most commonly prescribed drugs. However, pharmacological targeting of these receptors is limited by the numerous pathways under control of single receptors and even the ability of NRs to regulate multiple genes within pathways relevant in pathophysiological states. Thus, further attempts to exploit these receptors in drug development will rely upon increased efforts to characterize their molecular mechanism of action.

1.1 Classes of Nuclear Hormone Receptors

The nuclear hormone superfamily can be divided into four distinct classes which include the Class I steroid receptors, the Class II retinoid X receptor (RXR) heterodimers, the

Class III homodimers and the Class IV monomers. Overall, the segregation of the receptors into these classes is largely indicative of their unique DNA binding properties, ligands, and dimerization status on target genes (Mangelsdorf et al., 1995). Specifically, distinction among the classes is based upon relative similarities within the DNA-binding domain (DBD) and the ligand-binding domain (LBD). Class III and Class IV receptors include a significant number of orphan NRs, for which a putative physiological ligand has not yet been identified.

1.2 The Modular Structure of Nuclear Hormone Receptors

Nuclear hormone receptors are conserved in all metazoan species but are absent in plants and yeast (Figure 1.1). Indeed, the initial discovery of the ER α and GR receptors and the high degree of similarity observed provided profound evidence of the modular nature of these transcription factors (Green et al., 1986; Hollenberg et al., 1985). The most critical and highly conserved domains include the N-terminal DNA-binding domain (DBD), also termed the C domain (Luisi et al., 1991), and the α -helical C-terminal ligand-binding domain (LBD), or E domain, which functions to recognize ligand and for some receptors, promote dimerization (Wurtz et al., 1996). The extreme N-terminal A/B domain contains a flexible and highly variable region termed the AF-1 or activation function-1 domain. For some receptors, including PPAR γ and AR, this region demonstrates autonomous activation function through recruitment of activating cofactors which may be independent of ligand binding (Hu et al., 1996; McEwan, 2004). Situated between the DBD and the LBD is the hinge region or D region, which is poorly conserved and highly

variable both in terms of molecular structure as well as amino acid length. Altogether, the receptor functions by interaction with coactivators or corepressors that specifically associate with DNA-bound receptors depending upon the Apo or ligand-bound state of the receptor.

1.2a DNA-binding domain and NHR Target Sequence Recognition

Nuclear hormone receptors can directly influence gene transcription by binding to unique hormone response elements (HRE) which are functionally associated with their target genes (Beato, 1989). Typically, HRE are hexa-nucleotide sequences of 5'-AGGTCA-3' or 5'-AGAACA-3', termed half-sites, which are spatially oriented as direct, inverted or everted repeats or simply as monomeric sequences. Recognition of the half site is coordinated by two highly conserved zinc finger motifs which are unique to NRs. The zinc finger motif of the DBD is a small α -helical region which requires zinc ion association for proper protein conformation as well as to confer DNA-binding via charge interaction. The N-terminal zinc finger motif forms a recognition helix which binds the major groove of DNA and via the three-nucleotide P-box confers DNA sequence specificity, whereas the second zinc finger motif is hypothesized to promote homodimerization of steroid NR (Umesono and Evans, 1989)s. Overall, the DBD also contributes to appropriate dimerization pairing of the receptors, which for some receptors, including Rev-erb, is critical for efficient binding to cofactors (Harding and Lazar, 1995; Mader et al., 1993; Zamir et al., 1997b). Furthermore, a third α -helix, immediately adjacent to the DBD and contained within the hinge region, termed the C-

terminal extension (CTE), may contribute to relative DNA binding affinity and specificity (Harding and Lazar, 1995; Wilson et al., 1992; Zhao et al., 1998).

In addition to the high degree of similarity within the DBDs (~50% across all receptors), the HRE themselves vary little in terms of sequence. Overall, with the exception of the steroid receptors, which utilize 5-AGAACA-3', 5'-AGGTCA-3' is the most commonly utilized element. A first-order level of specificity is derived from the unique spacing and orientation of the half-site repeats. In the case of direct repeats, the spacing between repeats comprises a small number of nucleotides, typically a 1-5 nucleotide spacing. This spacing is defined according to the number of nucleotides found between the direct repeats and is referred to as DR2, DR4, etc. Upon the systematic, in vitro and unbiased determination of the binding site preferences for a series of RXR heterodimers and NR homodimers, the so-called "1 to 5" rule was described (Mangelsdorf and Evans, 1995). Subsequent structural and biochemical studies further clarified the basis for HRE binding specificity as well as other overall architecture of the receptor:DNA complex. For most RXR heterodimers, it was determined that the RXR binds at the 5' response element whereas the heterodimer partner binds 3' in a head-to-tail spatial orientation (Rastinejad et al., 1995). Incidentally, this is not the case for PPAR-RXR heterodimers, which bind to DR1 elements and the RXR molecule is 3' (Chandra et al., 2008). In either case, the heterodimer partner is regulated by the relative steric availability conferred by the half-site spacing. Thus, the spacing dictates both receptor:DNA specificity as well as RXR heterodimer pairing. Subtle alterations in spacing have been demonstrated for Rev-erb homodimers, which bind to a DR2 element, to induce steric hindrance which completely abrogates binding.

Structural studies of receptor DBD:DNA complexes for TR-RXR as well as Rev-erb homodimers also provided evidence for additional sites of protein-DNA interaction and specificity. RXR-TR structures revealed the importance of the CTE in DNA sequence binding specificity by contacts with the DNA minor groove (Rastinejad et al., 2000). Rev-erb homodimer:DNA crystal structures confirmed that indeed Rev-erb also utilized a CTE to promote homodimer cooperative binding and increased DNA binding specificity. Furthermore, Rev-erb utilizes a GRIP box element, which provides contacts with an A/T-rich nucleotide region upstream of the AGGTCA half-site (Zhao et al., 1998). Quite recently, the crystal structure of a (DBD-hinge-LBD) PPAR-RXR:DNA ternary complex was reported as well as functional data comparing GR:DNA binding-activity relationship (Chandra et al., 2008). Increasingly, these and other studies support a role for the DBD allosterically modulating receptor activity, by potentially affecting AF-1 function as well as cofactor interactions (Lefstin et al., 1994; Meijssing et al., 2009). Thus, the role of the DBD may extend well beyond DNA sequence recognition.

1.2b Ligand Binding Domain

The carboxy-terminal ligand-binding domain is a ligand-dependent molecular switch, which through its Activation Function-2 (AF-2) activity serves as a platform for interactions with various cofactors, depending on ligand status. Structurally, the ligand binding domain is globular and displays a three-layer α -helical “sandwich” comprised of

12 α -helices and commonly, two or three β folds. The architecture of the LBD results in a hydrophobic pocket typically formed by helices 5, 7 and 10, which can accommodate ligand (Egea et al., 2000). The size of the ligand pocket varies tremendously across the family of NHRs. For example, Class I steroid hormone receptors have small volume ligand pockets, on the order of ~500 cubic angstroms and bind ligands with high specificity and low nanomolar affinity. In contrast, other NRs such as the PPARs and PXR display large pockets up to 1600 cubic angstroms, which accommodate larger, more structurally diverse ligands such as phospholipids and xenobiotics with decreased specificity and much lower micromolar affinity (Nagy and Schwabe, 2004).

The surface of the ligand binding pocket comprised of helices 5, 7 and 10 makes specific contact with the ligand via key hydrophobic interactions and hydrogen binding. In the case of TR, the ligand is completely buried in the pocket and makes as many as eight specific contacts with the LBD (Wu and Koenig, 2000). Indeed, the relative degree of hydrophobic “burying” as well as the number of contacts made between ligand and receptor can correlate to the relative K_d , or “off-rate” of ligand with receptor. Once the receptor becomes engaged with ligand, key allosteric changes in receptor conformation are induced. Most prominently, there is a complete re-orientation of helix 12 which is critical for the AF-2 function of the LBD. Helix 12 shifts from its unliganded position at a 45degree angle away from the receptor, to contact the core LBD and form surface contacts with the ligand itself via a charge clamp mechanism (Hu and Lazar, 2000) . Additionally, helices 3-5 form a hydrophobic groove on the LBD surface which contacts

an α -helical hydrophobic leucine/isoleucine-rich motif is commonly found in coregulator molecules (Hu and Lazar, 1999; Hu et al., 2001; Nagy et al., 1999b; Nagy and Schwabe, 2004). Regions of the LBD also contribute to receptor dimerization. Helix 9 and 10 in particular, contain hydrophobic as well as key polar residues which promote specific protein-protein interactions between receptor LBDs.

Recent structures and supporting biochemical studies have also prominently demonstrated the inducible nature of the LBD ligand pocket. For example, crystallization of apo-receptors including Rev-erb β demonstrated the presence of bulky side chains from hydrophobic residues occluding the proposed ligand binding pocket (Woo et al., 2007). However, it was later shown that Rev-erb receptors can bind the large, planar heme molecule (Pardee et al., 2009a). Thus, there may be more plasticity in the LBD structure in accommodating physiological or synthetic ligands than previously appreciated.

1.2c A/B Domain and Ligand-Independent Activation Function-1 Activity

Across all nuclear hormone receptors, the N-terminal A/B domain constitutes the most variable region both in terms of sequence homology and amino acid length. NMR studies to deduce structural confirmation of numerous receptors show this region to be highly unordered and variable independent of ligand (Chandra et al., 2008). The receptors for which the A/B domain may have an activation function include the steroid hormone receptors (He et al., 2004). Interestingly, these receptors have the longest A/B

domain in terms of amino acid length and in some cases, have been observed by NMR to become increasingly ordered by DNA binding. In the case of androgen receptor for example, the A/B domain was shown to have autonomous, ligand-independent activation function which was critically mediated by a sequence of α -helical leucine-rich hydrophobic residues. Presumably, these residues are interacting with cofactors via hydrophobic protein-protein interactions to influence AR transcriptional activity. Additionally, the A/B domain of PPAR γ was demonstrated in several reports to be subject to MAPK phosphorylation, which markedly affected the activity of the receptor in 3T3-L1 cells (Hu et al., 1996). The physiological relevance of the phosphorylation status at this MAPK site was further shown to have affects on body weight and glucose metabolism in mouse knock-in models mimicking the phosphorylated form of the receptor (Rangwala et al., 2003). Thus for many receptors, this region appears to play a role in influencing the overall transcriptional activity.

Recently, for receptors such as Rev-erb α , this A/B region has been shown to be critical for protein stability. Phosphorylation of the Rev-erb α A/B domain by the kinase GSK-3 β stabilizes the receptor, preventing proteasome-induced degradation (Yin et al., 2006). Inhibition of GSK-3 β activity pharmacologically by lithium as well as manipulation of kinase levels by siRNA knockdown, dramatically reduced Rev-erb α protein levels in a proteasome-dependent manner. Since Rev-erb α is a critical component of the core circadian clock, and its protein levels are known to vary in a biphasic manner over the

24-hour cycle, regulation of this receptor within the A/B domain plays a very significant role in its molecular function.

In addition to phosphorylation, other post-translational modifications have been increasingly described to occur within this region, including sumoylation (Ghisletti et al., 2007) . These modifications may couple NHR with cellular and physiological signals in a temporal or tissue-specific manner. Modification of receptors within this domain would be hypothesized to alter protein-protein interactions, for example, to directly affect receptor activity.

1.2d Hinge/D Domain

The hinge domain, which varies in length among receptors, has long been hypothesized to function as an unordered, flexible domain linking the DBD to the LBD. For receptors such as GR, it contains the nuclear localization signal and can be a site of post-translational protein modification (Tsai and O'Malley, 1994). The recent X-ray crystal structure of Δ -A/B-PPAR γ and Δ -A/B-RXR α has shed some light into the structure of this particular domain. In these studies it was uniquely demonstrated that beyond the aforementioned α -helical CTE, the hinge domain is highly unordered and by deuterium exchange assay, is in a highly unordered state independent of the ligand status, including the examined apo-, partial-agonist of full-agonist-bound states (Chandra et al., 2008). However, it must be noted that for PPAR γ , the hinge domain is extremely small and that

for receptors such as Rev-erb, where the domain exceeds 200 amino acids, it may have an allosteric function, which may be governed by post-translational modifications or DNA/ligand binding. Other recent studies have indicated that the hinge region may be post-translationally modified by acetylation and other modifications, dramatically affecting the transcriptional activity of the receptor, as in the case of FXR (Kemper et al., 2009).

2. Nuclear Hormone Receptor Mechanism of Action

Ligands serve as molecular messengers of physiological status. Alterations in endocrine signaling or metabolic parameters, for example, may lead to dramatic changes in endogenous ligand concentrations. NRs initiate a response to these changes in ligand levels by coordinately regulating target genes and pathways to rapidly restore homeostasis (Yang et al., 2007). The ability of ligand to direct gene transcription is largely based on the principle that the liganded receptor promotes coactivator binding whereas the apo- or unliganded receptor binds corepressors. Coactivator and corepressor molecules in turn serve as molecular scaffolds to recruit complexes containing histone acetyltransferase or deacetylation activity, respectively, which drive chromatin remodeling at their associated target genes to induce gene activation or repression. In addition, to ligand-dependent cofactor recruitment, NR activity can also be affected by sequence-specific response element properties, ligand-induced translocation, and ligand-induced DNA binding.

2.1 Allosteric Effects of DNA Binding

Intriguingly, new data have revealed that hormone response elements may function as allosteric regulators of NR function. Relative activation levels among a panel of endogenous glucocorticoid response elements in the presence of ligand show dramatic variation, despite differences in sequence of as little as 1 nucleotide (Meijsing et al., 2009). Interestingly, there is no correlation in GR DNA binding activity, such that response elements with low intrinsic activity demonstrate robust DBD binding comparable to highly active RE. Structural, mutational and knockdown analysis revealed that subtle alterations in GRE sequence could induce conformational changes in the GR DBD which can subsequently affect cofactor recruitment or activity. Thus, independent of ligand, response element sequences are hypothesized to function as allosteric modulators of NR activity. Future studies may reveal if this phenomenon is generalizable to other NRS.

2.2 Ligand Effects on Subcellular Localization and DNA Binding

Class I nuclear hormone receptors, which bind steroidal ligands, include the glucocorticoid receptor (GR), androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), and mineralocorticoid receptor (MR). They are unique in that in that in the absence of ligand they are localized to the cytoplasm and bound to molecular chaperones such as heat shock proteins. Despite the tight association with chaperoning complexes, these receptors are still highly accessible to ligand binding. Ligand binding promotes dissociation of the chaperone complex, translocation to the

nucleus, homodimerization and association on palindromic response elements with 3-base pair spacing (Beato et al., 1995). For all other NRs, DNA binding is constitutive and independent of ligand.

2.2 Ligand-Mediated Regulation of LBD Activity

For a number of nuclear hormone receptors including TR, LXR and RAR it has been demonstrated that in the absence of ligand, target genes are actively repressed below a basal level (Baniahmad et al., 1992; Hartman et al., 2005; Horlein et al., 1995; Hu et al., 2003). In contrast, ligand association incrementally promotes both relief of repression to the basal level as well as dramatic increases in target gene activation. Thus, the difference in gene expression can be on the order of several magnitudes. In this way, the LBD can function as a “molecular switch” to dramatically affect target gene expression (Figure 1.3).

Isolation of peptides differentially associated with unliganded TR and RAR receptors by yeast two hybrid screening methods led to the discovery of the corepressors NCoR and SMRT (Chen and Evans, 1995; Horlein et al., 1995). Mapping of the RID revealed the presence of C-terminal receptor interaction domains in both coactivators and corepressors. Further studies isolated the specific motifs within the RIDs, LxxLL for coactivators, and IxxII for corepressors, referred to as NR boxes (coactivators) and CoRNR boxes (corepressors), respectively, which were required for binding (Darimont et

al., 1998; Hu and Lazar, 1999; Nagy et al., 1999b; Nolte et al., 1998; Shiau et al., 1998) . Structurally, these NR and CoRNR motifs were demonstrated to bind overlapping regions of the LBD comprised of helix 3, 4, 5, and sometimes 12. Differential binding between coactivators and corepressors is due to the ligand-induced re-positioning of helix 12 and the formation of a charge clamp between coactivator and helices 3 and 12, which sterically occludes corepressor binding (Figure 1.4). Proof that that these structural changes ascribed to helix 12 underlies cofactor selectivity is the demonstration that constitutively active receptors such as LRH-1 and Nurr1 adopt an LBD structure in which helix 12 is stably in the active conformation. On the contrary, Rev-erb, which lacks helix 12, constitutively associates with corepressors. Thus, ligand-induced alterations in the conformation of the LBD underlie differential recruitment of coactivators versus corepressors in a ligand-dependent manner.

2.2a Coactivators

A large number of coactivators, in excess of 200, have been indentified in both genetic screens (yeast two hybrid and others) as well as by proteomic co-precipitation methods. These cofactors bind in a ligand-dependent manner and in co-transfection or knockdown studies affect ligand-dependent activity of their cognate receptors. Increases in transcriptional activity associated with binding can be attributed to autonomous enzymatic activity, primarily histone acetyltransferase (HAT) activity, or the ability to recruit additional cofactors containing HAT activity, which direct local covalent histone modification. Among the first coactivators identified were those of the p160 family,

which were shown to bind TR, RAR and ER in a ligand-sensitive manner (Onate et al., 1995). The p160 family of proteins possesses relatively weak HAT activity. Other coactivators, including members of the CBP/p300 family, which have robust HAT activity, were later identified (Chen et al., 1999b). In contrast to the p160 family, these cofactors are recruited and bound indirectly in large activation complexes. In addition to HAT activity, histone methyltransferases such as CARM-1 function as coactivators and similarly modify the chromatin template by arginine and lysine methylation to induce gene activation (Chen et al., 1999a).

Additional coactivator complexes are reported to bind to liganded receptors which include cofactors with ATP-dependent nucleosome remodeling activity. In particular, recruitment of Swi/Snf proteins have been described for numerous NRs (Wade and Wolffe, 1999). ATP-dependent chromatin remodeling promotes repositioning of nucleosome structures to allow access for additional histone modifiers as well as drive association of basal transcription machinery to induce gene activation. Thus, in addition to covalent histone modifications to alter the histone code, these coactivators can enhance target gene expression by higher order chromatin remodeling.

Recent genome-wide studies to examine transcription factor occupancy utilizing chromatin immunoprecipitation (ChIP)-CHIP and ChIP-SEQ approaches coupled to gene expression assays have provided further insight into the mechanism of gene activation by NR at target genes (Carroll et al., 2006). For NRs including ER α , additional

transcription factors, or so-called “pioneer factors” were shown to colocalize to NR response elements (Carroll et al., 2005). Binding of these factors appears to be required for robust activation of a large number of target genes (Zhang et al., 2005). Hypotheses for the function of pioneer-factor recruitment include the fully synergistic recruitment of chromatin modifiers to induce transcription, or to initiate chromatin remodeling upstream of ligand-induced NR activation. It is unclear if other NRs utilize neighboring transcription factors in this manner to fine-tune the regulation of target genes (Zhang et al., 2005).

2.2b Corepressors

Prior to the discovery of the corepressors NCoR and SMRT, it was recognized that nuclear hormone receptors could actively repress transcription below basal levels in the absence of ligand. Through mutational analysis, it was known that regions of the LBD were required for repression (Casanova et al., 1994; Tong et al., 1996). The subsequent identification of NCoR (originally described as receptor interacting protein 13, RIP13) and SMRT (originally described as TR-associated cofactor TRAC) revealed that these large, highly analogous proteins functioned as molecular scaffolds for recruitment of other cofactors with histone deacetylase activity. Although additional corepressors have since been described, including RIP140 and others, it is clear that there are very few corepressors utilized by NRs when compared to coactivators, for which over 200 have been identified (Wei et al., 2000).

Antagonism of these NRs stably repositions helix 12 in a manner that cannot be achieved in the absence of ligand. This antagonist-driven repositioning of helix 12 promotes strong corepressor association, which generally requires binding of a potent, full agonist to displace. A number of pharmacological antagonists have been developed for clinical use. Tamoxifen, an antagonist for ER, takes advantage of relative NCoR and SMRT expression differences in various tissues to mechanistically function as a selective modulator. That is, in target tissues containing high levels of corepressors, antagonized ER has strong repression function, whereas in tissues with limited corepressor expression, minimal repression of target genes occurs (Osborne et al., 2000; Shang and Brown, 2002). Thus, for antagonists as well as partial agonists, it is possible to pharmacologically target various tissues and pathways in a gene-selective manner depending upon the relative expression levels of corepressor as well as the relative affinities for corepressor that the ligand or antagonist induces.

NCoR/SMRT

SMRT and NCoR corepressors are critical proteins for control of gene transcription. Genetic deletions of NCoR and SMRT in mice have shown that they are required for life (Jepsen et al., 2008; Jepsen et al., 2000). NCoR and SMRT are encoded by separate loci, but share a similar modular structure, possess over 45% amino acid homology, and function using the same mechanism (Figure 1.3). NCoR and SMRT are high-molecular weights protein of approximately 270kD which are ubiquitously expressed, with some exceptions. The liver contains higher NCoR levels whereas thymocytes predominantly

express SMRT. Alternative splicing of NCoR and SMRT can occur, which generates alternate protein products including SMRT τ , Rip13a and RIP13 Δ 1, but the expression profile or function of these alternate forms is not fully understood (Privalsky, 2004).

NCoR and SMRT share many modular functional domains (Privalsky, 2004). The N-terminus contains several repression domains (RDs), for which autonomous repression function can be demonstrated in reporter assays utilizing Gal-DBD fusions containing these regions. The proteins that can associate with these RDs include HDAC3, GPS2, TBL1 and TBLR1 (Guenther et al., 2000; Wen et al., 2000). The C-terminus contains several receptor interaction domains (RIDs), which contain hydrophobic, α -helical CoRNR motifs which can dock to the hydrophobic surface of NRs. Thus, the RIDs serve to facilitate ligand-sensitive and specific binding to NRs, whereas the N-terminal repression domains initiate formation of a high-molecular-weight complex containing HDAC activity.

Among the C-terminal repression domains, which NCoR has two and SMRT three, preference for NR binding partners can be observed. For example, TR, Rev-erb and LXR have been shown to prefer NCoR (Hu et al., 2003; Ishikawa et al., 1990; Ishizuka and Lazar, 2003; Zamir et al., 1997b), whereas RAR preferentially binds SMRT (Harding and Lazar, 1995). Even further, amino acids adjacent to the N-terminus of the CoRNR motif contained in the RID can promote both preference for NCoR versus SMRT, as well as

preference for a particular repression domain. TR, for example, prefers NCoR RD1 and RD2. In contrast RXR is permissible in terms of both corepressor association and RD domains. In this way, dimerization of the receptors results in a stoichiometry of one corepressor molecule (NCoR or SMRT) associated with a DNA-bound dimer.

NCoR and SMRT can repress genes both by competing with coactivators for binding as well as by active recruitment of additional repressive cofactors. Immunopurification of SMRT and NCoR-containing corepressor complexes demonstrated stoichiometric association with HDAC3 and the WD-40 repeat containing proteins TBL1, TBLR1 and GPS2 (Guenther et al., 2000). The core corepressor complex contains potent deacetylation activity on histone tails; however, recombinant HDAC3 contains negligible deacetylase activity. Newly translated HDAC3 is bound by the molecular chaperone Hsp90, and becomes associated with SMRT/NCoR via a transient, ATP-dependent association with the TRiC complex (Guenther et al., 2002). SMRT and NCoR function as activating cofactors of HDAC3. NCoR/SMRT regions required for HDAC3 activation were mapped by deletion mutagenesis to regions previously described as having autonomous repression function. NCoR/SMRT contain deacetylase activation domains, or DADs, which contain SANT motifs (Guenther et al., 2001). Indeed, the DAD motif is necessary and sufficient for HDAC3 activation. Further examination of the DAD domain demonstrated the requirement for Tyrosine 478 in HDAC3 activation (Ishizuka and Lazar, 2005). The requirement of the DAD domain and specifically Y478 in mediating HDAC3-dependent NCoR repression was confirmed in NCoR DAD^m mice,

which displayed dramatic decreases in gene repression of well-characterized NCoR target genes (Alenghat et al., 2008). Further studies also defined a role for a second SANT motif contained with the N-terminal RDs, termed the histone interaction domain (HID). This motif increased corepressor affinity for deacetylated histones, suggesting that the HID functioned in a feed-forward mechanism to further stabilize association of the core corepressor complex at target genes (Yu et al., 2003).

The ability of HDAC3 to repress gene transcription is related to its ability to affect the local chromatin environment. In vivo, 147 base pairs of DNA are packaged around an octamer of core histone proteins (two copies each of H2A, H2B, H3 and H4) which constitutes a nucleosome, and organized into higher order structures to facilitate packing of the DNA into the nucleus. Thus, accessibility of the DNA to transcription factors and the general transcription machinery is tightly regulated. In response to upstream signaling events, ATP dependent reactions including histone modifications and nucleosome repositioning occur to alter what is otherwise a chromatin structure that is highly repressive and structurally inaccessible (Berger, 2007). Subtle changes in the chromatin structure by these events results in an “opening” of the compacted DNA, rendering a more exposed chromatin structure that generally results in gene activation. To some extent, histone hyperacetylation by HATs neutralizes the positively-charged lysine residues on which these modifications occur, resulting in decreased charge interaction with the negatively-charged DNA, thus loosening the tight nucleosome to DNA interaction (Strahl and Allis, 2000). In contrast, hypoacetylated histone structures

resulting from HDAC activity increase the charge contacts between DNA and the histone tails, which closes the chromatin structure and is generally repressive (Lee and Workman, 2007).

However, within the last decade, it has been recognized that histone modification of chromatin occurs in patterns, known as the “histone code”, to allow for tight regulation of gene transcription in response to developmental, spatial and temporal cues (Strahl and Allis, 2000). Multiple post-translational modifications including acetylation, methylation, ubiquitination, sumoylation, and phosphorylation can occur on histone tails. These modifications can occur on similar residues of the histone tails, so that in effect there are “competing” modifications. In addition, the level of modification can be mono, bi or tri-valent, with varying outcomes on transcription. Generally, tri- methylation of H3K4 in the promoter regions is thought to be associated with active genes (Ruthenburg et al., 2007), whereas H3K27 methylation, driven by Polycomb group proteins is repressive, and the associated genes are generally inactive (Trojer and Reinberg, 2006). However, this simplistic view does not reflect the combinatorial effects of various histone modifications. For example, several genes important in development are found to contain both activating and repressing marks and thus have bivalent modification (Bernstein et al., 2006). This may represent a priming mechanism for the temporal control of gene transcription. Furthermore, the temporal progression of modifications leading to transcriptional activation and repression are still being elucidated. HDACs, for example, which can rapidly alter lysine modification status, could function early in the sequence, to

deacetylate and “reset” lysine status for methyltransferases. Or, in contrast, HDACs could function late in the pathway, to rapidly expose the positive lysine charge of the histone tails and stimulate rapid histone:DNA interaction.

3. Physiological Functions of LXR

LXR α (NR1H3) and LXR β (NR1H2) are members of the nuclear hormone receptor superfamily (Janowski et al., 1996). Their physiological ligands are oxysterols, including oxidized LDL (oxLDL) and cholesterol metabolites including 24(*S*),25-epoxycholesterol and 22 (*R*)-hydroxycholesterol (Willy et al., 1995). LXR α is highly expressed in the liver, and to a lesser extent in the intestine, adipose tissue, macrophages, lung, kidney, and adrenal glands, whereas LXR β has ubiquitous expression. LXRs, together with RXR, have been shown to have important roles in cholesterol homeostasis by regulating cholesterol metabolism and reverse cholesterol uptake. These receptors have a pronounced protective role in atherogenesis, which is thought to be derived from the ligand-sensitive induction of the reverse cholesterol transporters of the ABCA family as well as the modulation of the inflammatory response in macrophages (Joseph et al., 2003; Joseph et al., 2002; Levin et al., 2005).

Macrophages play important roles in various immune functions in secretion of potent inflammatory factors such as cytokines. Atherosclerosis is a chronic inflammatory disease and a pathological process in which the pro-inflammatory activities of

macrophages are well documented. The nuclear receptor LXR has been shown to have a negative role in regulating these inflammatory events (Tangirala et al., 2002).

It has been demonstrated that there is a reciprocal repression, or transrepression, of inflammatory target genes such as *iNOS*, *COX-2*, *IL-6* and *IL-1 β* following exposure to pro-inflammatory stimuli such as LPS, TNF- α , IL-1 β in the presence of LXR agonists (Joseph et al., 2003). In addition to the genes described, large subsets of inflammatory and innate immune response genes were characterized by microarray as negatively regulated by LXR transrepression (Ogawa et al., 2005). Furthermore, transrepression was shown to be isoform independent, as transrepression was competent in wild-type, *LXR α* ^{-/-}, *LXR β* ^{-/-} mouse macrophages, and only abolished in *LXR $\alpha\beta$* ^{-/-} macrophages (Joseph et al., 2003).

Mouse models of the anti-inflammatory properties of LXR agonists further demonstrate the clinical implications of their use as pharmacological agents. Intraperitoneal injection of LPS in *LXR $\alpha\beta$* ^{-/-} mice leads to exaggerated systemic cytokine response and expression (Joseph et al., 2003). Mouse models have also demonstrated anti-inflammatory roles for LXR agonists in cutaneous inflammation and allergic dermatitis (Tontonoz and Mangelsdorf, 2003). Finally, atherogenic inflammation was shown to be reduced upon treatment of hypercholesterolemic apoE^{-/-} mice with non-steroidal LXR agonist (Joseph et al., 2002).

The mechanism of ant-inflammatory transrepression associated with LXR is thought to be indirect and not due to recruitment of LXR to LXREs, but rather to proximal promoter regions of these pro-inflammatory target genes. A new mechanism of transrepression involving the ligand-dependent repression of inflammatory genes regulated by the PPAR- γ and LXR receptors has been proposed (Pascual and Glass, 2006). In this model, transrepression was shown to be dependent upon the presence of the corepressor N-CoR at pro-inflammatory target gene promoters such as *iNOS*. As *iNOS* is known to be repressed in the presence of PPAR- γ and LXR agonists, it was demonstrated that sumoylated LXR/PPAR- γ can stabilize an N-CoR-HDAC3-TBL1-TBLR1 corepressor complex at NF- κ B-bound target genes. Such stabilization occurs by sumoylation of LXR/PPAR- γ and subsequent inhibition by sumoylated LXR/PPAR- γ of the ubiquitination-mediated proteasomal degradation of the N-CoR repressor complex at the *iNOS* promoter (Ghisletti et al., 2007; Pascual et al., 2005). Thus, the ability of LXR to direct cholesterol metabolism as well as its potent-anti-inflammatory effects suggest that targeting of this receptor in cardiovascular disease may be of strong clinical value.

4. Physiological Functions of Rev-erb α

Rev-erb α plays a critical role in regulation of the core circadian clock. It directly represses transcription of the *Bmal1* gene in addition to regulating its own transcription, and thus serves to link the positive and negative limbs of the circadian clock. Knockout of the mouse Rev-erb α gene leads to a number of defects in reproduction and modest

effects on cerebellar development (Chomez et al., 2000). Due to the role of Rev-erb α in regulating the core clock, analysis of the physiological circadian rhythm by mouse wheel behavior indicates that indeed deletion of this receptor results in circadian abnormalities (Preitner et al., 2002). A number of Rev-erb α target genes have now been described, including genes known to regulate lipid homeostasis, glucose homeostasis, cholesterol metabolism and regulation of adipogenesis and adipokine expression (Duez and Staels, 2008) . There appears to be an intimate link between metabolic regulation and the core circadian clock (Kohsaka et al., 2007). Rev-erb α may participate both by its regulation of the core clock as well as its direct metabolic gene targets.

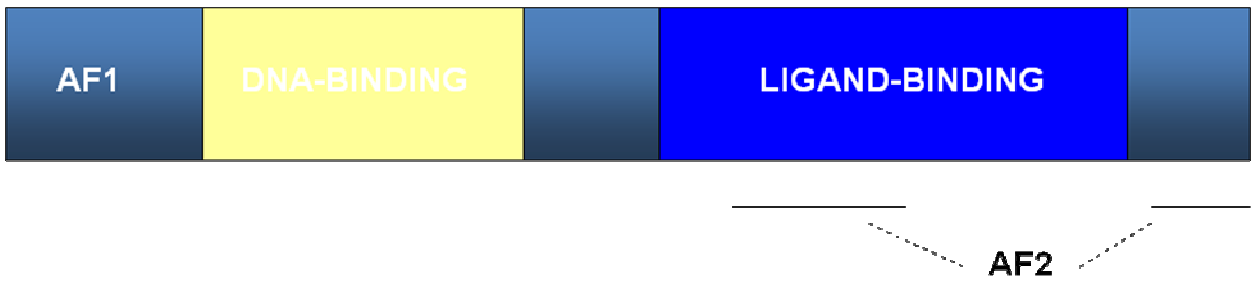


Figure 1.1. General schematic of nuclear receptor structure.



Figure 1.2. General schematic of corepressor modular domains. The red regions represent SANT domains 1 and 2. The RD1, 2 and 3 domains have autonomous repression function whereas the C-terminal repression domains interact with nuclear hormone receptors.

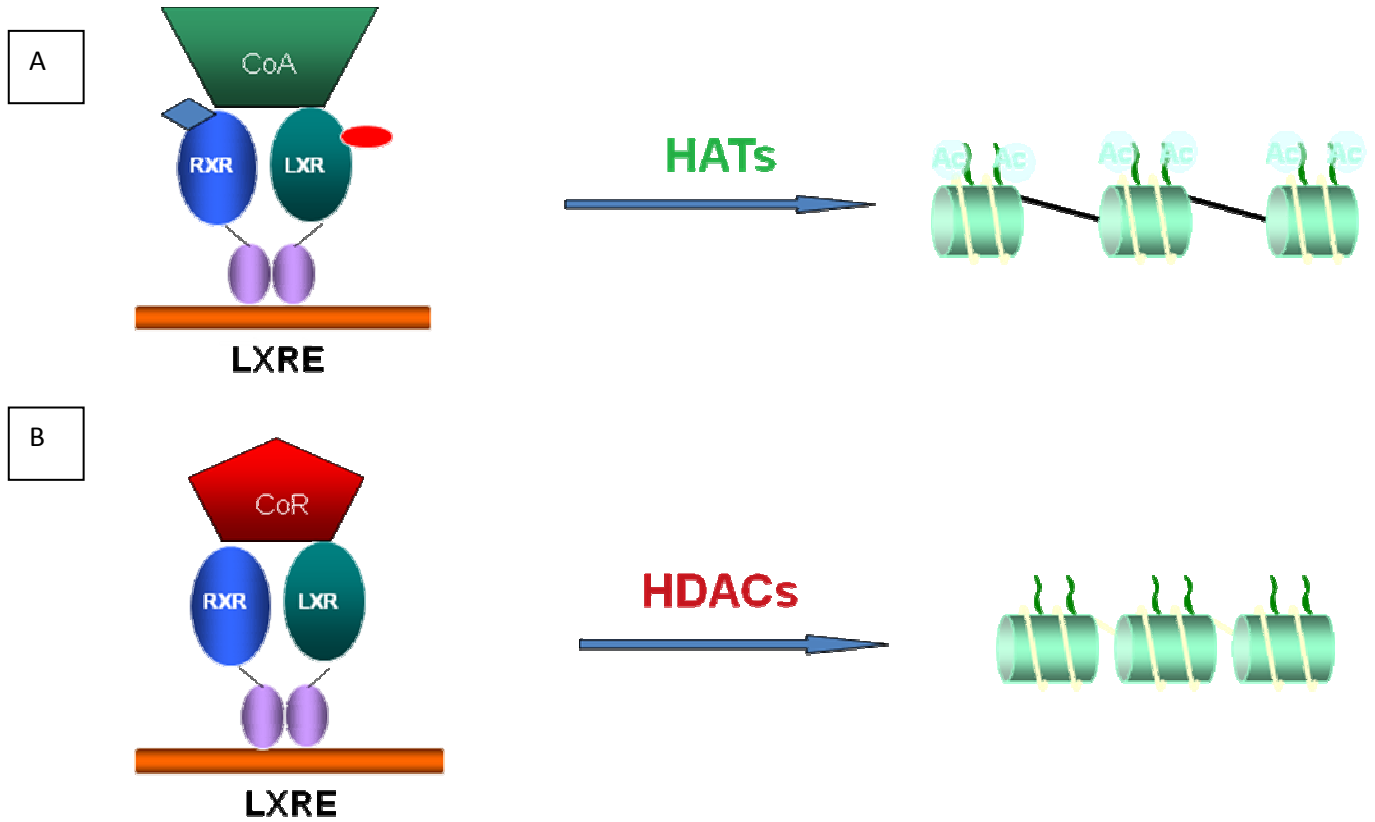


Figure 1.3 Mechanism of corepressor exchange. A. Represents the liganded NR state. The NR is associated with coactivators and HAT activity. B. Represents the unliganded NR state. The NR is associated with corepressor and HDAC activity.

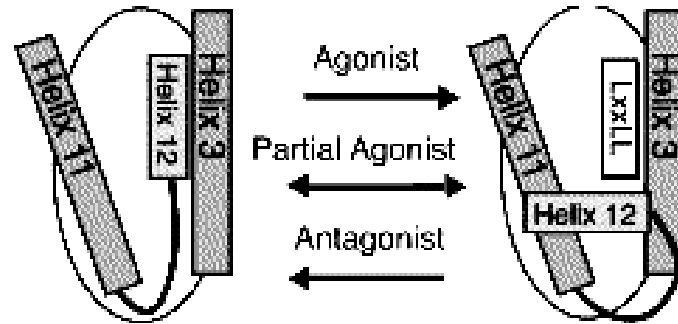


Figure 1.4 Binding of agonist to nuclear hormone receptors flips the “molecular switch”, or helix 12, to confer a conformation that exposes a hydrophobic cleft that promotes coactivator association via the LxxLL motif.

CHAPTER TWO

SELECTIVE PARTIAL AGONISM OF THE LIVER X RECEPTOR α IS RELATED TO DIFERENTIAL RECRUTMENT BY COREPRESSORS

I. INTRODUCTION:

The Liver X Receptors, LXR [nuclear receptor (NR1H3)] and LXR β (NR1H2) are highly homologous members of the nuclear hormone receptor superfamily that are encoded by two distinct genes (Willy et al., 1995). LXR displays a more limited expression profile, with high expression in the liver, adipose, intestine, kidneys, and macrophages, whereas LXR β is ubiquitously expressed. These receptors have been shown to play important roles in cholesterol and lipid homeostasis as well as innate immunity and inflammation. Although first described as an orphan nuclear receptor, LXR was later shown to be regulated by endogenous oxysterol ligands (Janowski et al., 1996; Lehmann et al., 1997). LXRs regulate transcription at target genes via binding to LXREs (liver X receptor response element) within promoters of target genes. In the unliganded state, LXR preferentially associates with the nuclear receptor corepressor (NCoR) (Hu et al., 2003). Upon ligand binding, the corepressor complex is dismissed and coactivators such as steroid receptor coactivator-1 (SRC-1) are recruited, facilitating alterations to local chromatin architecture and subsequent recruitment of the general transcription machinery.

LXR regulates whole-body cholesterol metabolism via direct transcriptional regulation of ATP-binding cassette transporter (ABC) A1 in macrophages, which promotes cellular cholesterol mobilization and efflux from macrophages to apolipoprotein A-1 and apolipoprotein E acceptors to form nascent high density lipoprotein particles (Repa et al., 2000). Together with other LXR target genes including apolipoprotein E, these proteins regulate a pathway for the removal of cholesterol from the periphery for transport to the

liver termed reverse cholesterol transport (Laffitte et al., 2001). Additionally, in rodents LXR- promotes the hepatic induction of cholesterol 7, which facilitates hepato-biliary cholesterol secretion in the face of excess dietary cholesterol (Chiang et al., 2001). This is dramatically illustrated by mice lacking LXR, which display severe hypercholesterolemia and hepatic steatosis on a high-fat, high-cholesterol diet (Peet et al., 1998).

Due to the potential of LXR as a drug target for dyslipidemia, several highly potent and active agonists have been synthesized, including T091317 (Schultz et al., 2000). However, the therapeutic utility of LXR ligands has been limited by hepatic steatosis and hypertriglyceridemia (Grefhorst et al., 2002; Schultz et al., 2000). These effects may be due to direct induction of the lipogenic transcription factor sterol-regulatory element binding protein 1 (SREBP1) as well as other lipogenic LXR target genes including fatty acid synthase (FAS), which leads to a robust induction of fatty acid synthesis in the liver. The limitations imposed by the dual regulation of *de novo* lipogenesis and reverse cholesterol transport by LXR indicate that a target-specific approach to modulation of LXR activity will be critical for successful therapeutic intervention. It is also critical to study LXR effects in humans because the beneficial induction of cholesterol 7 α may be rodent specific, and rodents also lack functional cholesteryl ester transport protein, which is a detrimental LXR target gene in humans (Luo and Tall, 2000)

In the case of estrogen receptor (ER), differential recruitment of coactivators and corepressors may explain why selective ER modulators (SERMs) have target gene and cell type selectivity (Shang and Brown, 2002). SERM binding induces ER conformations

that are different from those of ER bound to potent agonists. Similarly, different conformations have been noted for LXR bound to the partial agonist 24(S), 25-epoxycholesterol and the potent synthetic agonist T091317, suggesting that it may be possible to selectively modulate the activity of LXR (Williams et al., 2003). Selective modulation could involve quantitative or qualitative differences in coactivator association with liganded LXR (Quinet et al., 2004; Schmidt et al., 2006), (Jaye et al., 2005; Miao et al., 2004; Traves et al., 2007). Alternatively, although not mutually exclusively, different ligands could alter interaction with NCoR (Albers et al., 2006).

Here we show that NCoR binding plays a critical role in the differential response of LXR to a variety of ligands. Using *in vitro* biochemical studies and chromatin immunoprecipitation (ChIP) analysis in cells, we demonstrate that, independent of binding affinity, different LXR agonist ligands have vastly different properties in destabilizing the association of NCoR with LXR target genes. In addition, modulation of the level of NCoR protein differentially influences ligand activation of LXR and is target gene specific. Moreover, individual ligands have target gene-specific effects on NCoR dismissal from endogenous LXR targets. Thus, differential NCoR recruitment underlies gene-specific differences in activation by these ligands.

II. MATERIALS AND METHODS:

Reagents Compounds were provided by GlaxoSmithKline or obtained from Calbiochem (La Jolla, CA). DMSO was obtained from Sigma (St. Louis, MO). For Western blot analysis, the antibody for NCoR has been previously described, and antibodies for β -actin (Kuwabara and Sigman) and Ran (BD Biosciences, San Jose, CA) were obtained. For chromatin immunoprecipitation, antibodies for NCoR were obtained from Abcam (Cambridge, MA), and control IgG antibodies were from Calbiochem. Protein A-Sepharose was from Amersham Biosciences (Piscataway, NJ).

Plasmids pFA-CMV-Gal4-hLXR α ligand-binding domain (LBD) (162–447), pFA-CMV-Gal4-DBD, and pGL3-hABCA1-Luc were provided by GlaxoSmithKline; GAL-5XUAS-SV40 luciferase, β -galactosidase, pCMX-NCoR-FLAG, pCMX-mLXR α , and pCMX-mRXR α plasmids have been previously described. pGL3-basic was obtained from Promega (Madison, WI), and pCIS and pCIS-LXRE-Luc were obtained from Stratagene (San Diego, CA).

FRET Assay A modified polyhistidine tag was fused in frame to the human LXR ligand-binding domain and subcloned into the expression vector pRSETa (Invitrogen). The human LXR ligand binding domain was expressed in *Escherichia coli* strain BL21(DE3) in Rich PO₄ media with 0.1 mg/ml ampicillin at 25 C for 12 h, and 0.25 mM isopropyl- β -D-thiogalactopyranoside was added. Cells were resuspended and concentrated cell slurries

were stored in PBS at -80°C . Cell paste was resuspended in TBS, pH 8.0 (25 mM Tris, 150 mM NaCl). Cells were lysed by an APV Rannie MINI-lab homogenizer, and cell debris was removed by centrifugation. The cleared supernatant was filtered through coarse prefilters, and TBS, pH 8.0, containing 500 mM imidazole was added to obtain a final imidazole concentration of 50 mM. Lysate was loaded onto Sepharose (Ni⁺⁺ charged) Chelation resin (Pharmacia) and preequilibrated with TBS (pH 8.0)/50 mM imidazole. The column was washed with approximately one column volume of TBS (pH 8.0) containing 95 mM imidazole. LXR-LBD was eluted with a gradient from 50–500 mM imidazole. Column peak fractions were pooled immediately and diluted 5-fold with 25 mM Tris (pH 8.0), containing 5% 1,2-propanediol, 0.5 mM EDTA, and 5 mM dithiothreitol (DTT). The diluted protein sample was then loaded onto Poros HQ, washed with the dilution buffer, and the protein was eluted with a gradient from 50–500 mM NaCl. Peak fractions were pooled and concentrated using Centri-prep 10K (Amicon) filter devices and subjected to size exclusion, using Superdex-75 resin (Pharmacia) preequilibrated with TBS (pH 8.0), containing 5% 1,2-propanediol, 0.5 mM EDTA, and 5 mM DTT. LXR protein was diluted to in PBS, and 5-fold molar excess of NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) was added in a minimal volume of PBS and incubated for 30 min. The biotinylation modification reaction was stopped by the addition of 2000x molar excess of Tris-HCl, pH 8. The modified LXR protein was dialyzed against PBS containing 5 mM DTT, 2 mM EDTA, and 2% sucrose. The biotinylated LXR protein was subjected to mass spectrometric analysis until approximately 95% of the protein had at least a single site of biotinylation; and the overall extent of biotinylation followed a normal distribution of multiple sites, ranging from one to nine. The

biotinylated protein was incubated for 20–25 min at a concentration of 25 nM in assay buffer [50 mM KCl, 50 mM Tris (pH 8), 0.1 mg/ml fatty acid-free BSA; 10 mM DTT] with equimolar amounts of streptavidin-AlloPhycoCyanin (APC, Molecular Probes Inc., Eugene, OR). At the same time, the biotinylated peptide of SRC-1 or NCoR at a concentration of 25 nM was incubated in assay buffer with a molar amount of streptavidin-labeled Europium (Wallac, Inc., Boston, MA) for 20–25 min. After the initial incubations are completed, a 10 molar excess of cold biotin was added to each of the solutions to block the unattached streptavidin reagents. After 20 min the solutions were mixed yielding a concentration of 12.5 nM for the dye-labeled LXR protein and SRC-1 or NCoR peptide.

Mammalian Cell Culture and Transfection HEK-293T and HepG2 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and penicillin/streptomycin (Invitrogen) at 37 C in 5% CO₂. Transient transfection of 293T cells was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, cells were seeded in 24-well plates and allowed to adhere before transfection with 1.1 µg total of appropriate plasmids and 2.5µl of lipofectamine reagent in Opti-MEM (Invitrogen). Transfection mixture was incubated with cells overnight in DMEM containing 10% delipidated bovine serum (Intracel, Frederick, MD). The following day, the transfection mixture was aspirated and replaced with fresh delipidated medium. Cells were then treated with indicated compounds or vehicle in delipidated medium overnight for 16 h.

RNA Interference 293T cells were seeded into six-well plates and transfected with siRNA duplexes (Dharmacon, Lafayette, CO) targeting human NCoR or a nontargeting control using Lipofectamine 2000. Cells were then seeded in 24-well plates for further studies. HepG2 knockdown was carried out using the same siRNA duplexes, but cells were transfected using the Amaxa system, Cell Solution V, protocol T-28. For all knockdown studies, cells were harvested 96 h after knockdown for further analysis.

Luciferase Reporter Assay After compound treatment for 16 h, cells were lysed in 100 μ l of Passive Lysis Buffer (Promega) containing complete protease inhibitor (Roche, Indianapolis, IN). Cells were lysed by freeze-thaw at -80 C, and 5 μ l of lysate was used for luciferase assay (Promega) or β -galactosidase assay. Relative light units for luciferase were normalized to β -gal activity.

Reverse Transcription and Quantitative PCR Cells were lysed in Buffer RLT and processed using the RNeasy kit (QIAGEN, Chatsworth, CA) according to manufacturer's instructions. Isolated RNA was reverse transcribed with oligo-dT primers using either Sprint Power Script reagents (CLONTECH Laboratories, Inc., Palo Alto, CA) or SuperScript II (Invitrogen) according to the supplied protocol. cDNA was used in Taqman reactions using commercially available primer/probe sets (Applied Biosystems, Foster City, CA) and Taqman Universal PCR mix (Applied Biosystems). Quantitative PCR was performed on the Applied Systems 7900HT or 7500 Real-Time System. Data

were analyzed using the standard curve method with 36B4 serving as the housekeeping gene.

Chromatin Immunoprecipitation 293T cells were transfected as described in 10-cm dishes according to manufacturer instructions or HepG2 cells were plated in 10-cm dishes. Compounds were added in delipidated media overnight for 16 h. Cells were washed with PBS, and cell pellets were lysed in ChIP lysis buffer [50 mM HEPES/NaOH, 1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and complete protease inhibitor], incubated on ice for 10 min, and diluted in ChIP dilution buffer (50 mM HEPES/NaOH, 155 mM NaCl, 1.1% Triton X-100, 0.11% Na-deoxycholate, 1 mM phenylmethylsulfonylfluoride, and protease inhibitors with EDTA). Lysate was sonicated three times for 15 sec to yield an average DNA fragment length of approximately 500 bp. Lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4 C. HepG2 samples were precleared for 2 h at 4 C with protein-A-sepharose beads containing sonicated salmon sperm DNA. Lysates were then incubated overnight at 4C with the following antibodies: anti-NCoR (Abcam) or rabbit IgG (Calbiochem) as a negative control. Immune complexes were then precipitated with protein A-sepharose for 2 h at 4 C. Complexes were subsequently washed with low-salt buffer, high-salt buffer, lithium chloride buffer, and Tris-EDTA as described by Upstate Biotechnology, Inc. (Lake Placid, NY) with minor modifications. Complexes were eluted in SDS elution buffer, and cross-links were reversed for a minimum of 6 h at 65 C. Eluted DNA was processed either by phenol/chloroform

extraction or with the PCR Purification Kit (QIAGEN). DNA was used for quantitative PCR with POWER SYBR mix (Applied Biosystems) and the following primers: *hFASN*-forward (F)-gttactgccggtcatcgca, *hFASN*-reverse (R)-tctcgggtctgggtccc, *hABCA1*-F-acgtgctttctgctgagtga, *hABCA1*-R-accgagcgcagaggttacta, *hSREBF1*-F-tgctgcacccatattatctcc, *hSREBF1*-R-gctccaaccctccgtttact, SV40-F-ttccggtactgttgtaaaatgg, SV40-R accagggcgtatctcttcatagc, *h36B4*-F-acgctgctgaacatgctcaa, *h36B4*-R-gatgctgccattgtcgaaca. Upstream primer sequences are available upon request.

Immunoblotting Lysates were prepared in passive lysis buffer for cotransfection studies or in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.4% deoxycholate, 0.1% SDS]. Lysate (15–20 µg) was loaded on 4–20% Tris-Glycine gels (Invitrogen) and electrophoresed. Transfer was performed onto Immobilon-P (Millipore Corp., Bedford, MA) membranes, and membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20. Membranes were incubated overnight at 4 C with primary antibodies diluted in blocking buffer. Membranes were washed extensively and incubated with appropriate secondary antibodies (Pierce). After washes, chemiluminescent signal was detected with ECL-Plus (Amersham Biosciences, Arlington Heights, IL).

III. RESULTS

Partial Agonism of LXR

We studied two ligands that have been previously described, the sulfonamide T091317 and GW3965 (Schultz et al., 2002, Collins, et al., 2002), as well as a novel LXR ligand GSK418224 (Fig. 2.1A), referred to as T09, GW965, and GSK224. The activities of the ligands were tested by *in vitro* fluorescence-resonance energy transfer (FRET) assays, luciferase reporter assays using Gal-LXR or full-length LXR in human kidney 293T cells, and assays of endogenous gene expression in human liver cells. The results are summarized in Table 1. In transient transfections, T09 induced high levels of LXR transactivation, relative to the maximum efficacy of GW965, which was only approximately 23% of that of T09 (Fig. 2.1B). Maximum efficacy of GSK224 was an order of magnitude lower still and approximately 1% that of T09 (Fig. 2.1B, see *inset*). Thus, both GW965 and GSK224 functioned as partial agonists relative to the full agonist T09. To determine whether the reduced efficacies at saturating concentrations were due to differences in coactivator recruitment, a cell-free FRET assay was used to measure recruitment of a biotinylated SRC-1 NR box peptide (Heery et al., 1997) in the presence of increasing concentrations of ligand. Comparisons of the relative ability to recruit coactivator at maximal ligand concentration revealed only a minor difference between T09 and GW965, whereas the effectiveness of GSK224 was less but nevertheless considerably more than would have been predicted by its low relative level of transcriptional activity (Fig. 2.1C).

Differential Effects of Ligands on Target Gene Recruitment of NCoR by LXR

We next employed CHIP to assess the effects of LXR ligands on NCoR recruitment by Gal4-LXR in intact cells. As expected, NCoR was robustly recruited by Gal-LXR relative to the Gal DNA-binding domain (DBD) alone (Fig. 2.3). Consistent with the predictions from *in vitro* studies, GSK224 had little effect on NCoR recruitment, and GW965 had a moderate effect, whereas T09 led to near complete dissociation of NCoR from the reporter gene (Fig. 2.3).

LXR Transactivation Is Modulated by Cellular NCoR Levels

To determine the role of NCoR in modulating the activity of LXR ligands, we repeated the transactivation assays at saturating concentrations of ligands after a reduction in NCoR levels using small interfering RNA (siRNAs). Under these conditions, activation by GSK224 increased approximately 5.2-fold, and the activity of GW965 also increased markedly (3.7-fold, Fig. 2.4A). Even T09 activity was increased when NCoR levels were reduced, although the change was more modest (Fig. 2.4A). Over-expression of NCoR had its greatest effect on the activity of GW965, which was reduced by about 50% (Fig. 2.4B). GSK224 activity was decreased only approximately 1.3-fold, consistent with the previous conclusion that endogenous NCoR was already bound in this setting and the activity of the full agonist T09 was affected even less by exogenous NCoR (Fig. 2.4B). In sum, GW965-mediated association with NCoR was highly dynamic and thus most sensitive to the level of NCoR.

Partial Agonism of LXR/Retinoic X Receptor (RXR) Heterodimers

We next determined whether the ligands also differentially activated the full-length receptor LXR by cotransfection expression plasmids for LXR and RXR along with LXR-responsive luciferase reporter constructs into 293T cells. Using a reporter containing three copies of an LXR-response element (DR-4) cloned upstream of the herpes simplex virus thymidine kinase promoter, T09 and GW965 induced similar levels of activation, whereas GSK224 treatment led to a lower induction (Fig. 2.5A). We also examined a luciferase reporter driven by the human ABCA1 promoter. 293T cells were cotransfected with a luciferase reporter driven by 1.45 kb of the proximal human *ABCA1* promoter, containing the LXR-response element (Costet et al., 2000). *hABCA1*-driven luciferase activity was induced to the greatest extent by T09, followed by GW965, and then GSK224. Together, these results confirm that T09 is the strongest LXR agonist, with GW965 intermediate, and GSK224 a weaker agonist.

Partial Agonism of LXR in Human Liver-Derived Cells

We next determined the activity of these LXR ligands on endogenous gene expression in human HepG2 hepatoma cells. Fatty acid synthase (FAS), an LXR target that may contribute to hepatic triglyceride accumulation that is an undesirable side effect for clinical LXR agonism, was dramatically induced by T09, whereas GW965 was less active at inducing FAS mRNA and GSK224 even less so (Fig. 2.6A). By contrast, in the same cells, the expression of another lipogenic LXR target, SREBP1, was increased to similar extents by all three ligands (Fig. 2.6B). Similarly all three ligands activated another LXR

target, the cholesterol efflux gene ABCA1 (Fig. 2.6C). Thus, T09 behaved as a full agonist at all genes tested, whereas the degree of agonism of GSK224 and GW965 was gene specific.

NCoR Occupancy of Endogenous LXR Target Genes in Liver Cells

To further assess the contribution of NCoR association to relative expression levels at these LXR target genes, we performed ChIP in the vicinity of known LXREs in the endogenous FAS, SREBP1, and ABCA1 genes at saturating concentrations of each of the LXR ligands. The degree of NCoR dismissal from the fatty acid synthase gene was ligand specific and, consistent with the gene expression, reduced only modestly by GSK224, moderately by GW965, and markedly by T09 (Fig. 2.7A). By contrast, only a modest difference in relative NCoR recruitment was observed among the ligands at the SREBP1 promoter, consistent with the gene expression data (Fig. 2.7B). Examination of the LXRE at *ABCA1* revealed a trend of graded occupancy; however, the only significant difference observed was between GW965 and T09. Thus, the level of NCoR occupancy correlated with the differential activation of these LXR target genes.

Knockdown of NCoR Enhances Expression of LXR Target Genes

Given the gene-specific occupancy by NCoR in the presence of various LXR ligands, we explored the consequence of NCoR knockdown. Consistent with NCoR occupancy shown earlier, depletion of NCoR induced expression of the FAS gene (Fig. 2.8A). Reduction in NCoR levels also enhanced activation by GSK224 and GSK965, but not T09, which was

also consistent with the level of NCoR occupancy in the presence of these compounds (Fig. 2.8A). NCoR depletion also potentiated induction of SREBP1 and ABCA1 mRNA by GSK224, and in the case of ABCA1 activation was also increased, but to a lesser extent, by the other compounds (Fig. 2.8, B and C). Thus, the degree of association with NCoR in the presence of LXR ligands is a target gene-specific determinant of partial agonist activity.

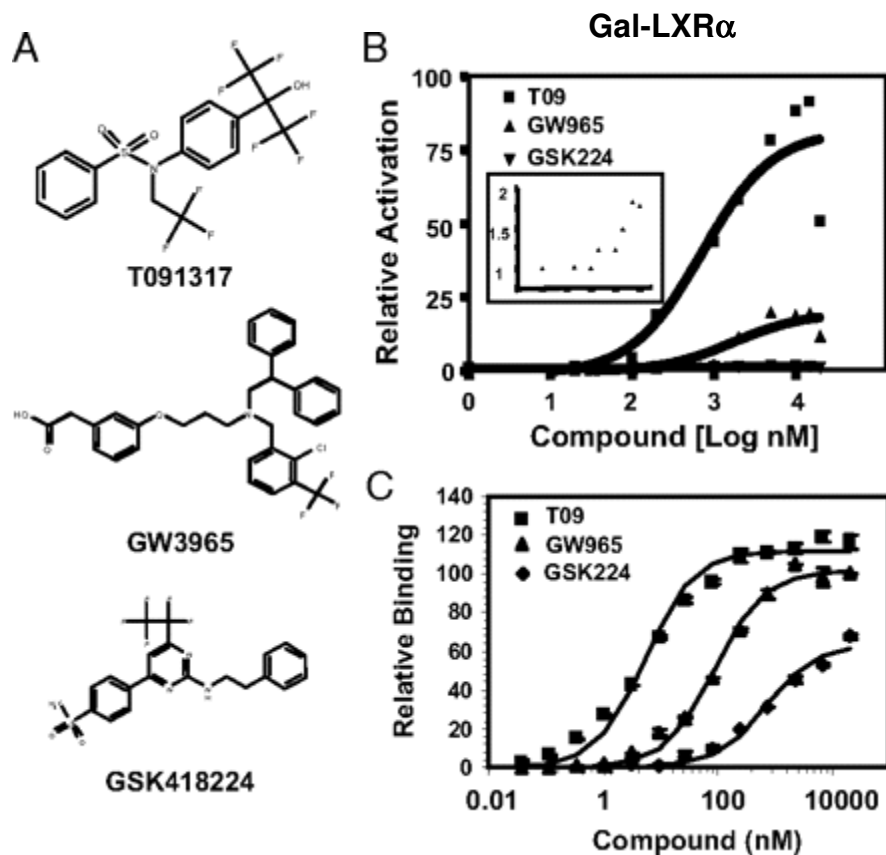
IV. DISCUSSION

We have described a mode of selective modulation of LXR in which the relative recruitment of the corepressor NCoR is a critical determinant of transcriptional efficacy. Two compounds that function as partial agonists relative to T09 fail to completely dissociate NCoR from LXR, with the least active compound causing almost no dissociation either in FRET assays performed *in vitro* or in ChIP assays in living cells. Affinity of liganded LXR for NCoR is likely to explain a portion of the reduced efficacy of these compounds. Consistent with this, removal of NCoR from cells enhanced the activity of the partial agonists. Others have previously correlated failure to dissociate NCoR with partial agonist activity (Albers et al., 2006). However, the present report is the first to demonstrate differential NCoR dismissal from endogenous genes and the first to test and confirm the NCoR hypothesis by manipulating NCoR concentrations.

It is likely that partial agonism reflects a combination of altered coactivator affinity as well as failure to completely dissociate NCoR. In this regard, it is of interest that SERMs differ from the full agonist estradiol because they alter the range of coactivator specificity as well as favoring interaction with corepressors (Shang et al., 2000). Note that, unlike LXR, ER does not have affinity for NCoR nor is it mostly bound to target genes in the unliganded state. Thus the role of corepressors is likely to be greater for LXR. This mechanism is potentially applicable to all NRs that bind corepressors, and particularly those that are bound to target genes in the absence of ligand (Hu et al., 1999).

For the development of potential therapies targeting LXR, the concomitant induction of hepatic *de novo* lipogenesis remains a major obstacle. Thus, it was of great interest to assess whether compounds that functioned as partial agonists *in vitro* and in transient transfection assays retained these properties on endogenous genes in liver cells. Indeed, GSK224 had very little effect on the lipogenic FAS gene, in part due to its failure to dissociate NCoR. Consistent with the differential effects of these ligands on NCoR dismissal at the FAS promoter, rodent studies have shown that indeed GW965 treatment results in decreased hepatic triglyceride accumulation compared with the full agonist T09 (Miao et al., 2004). However, GSK224 did activate the lipogenic SREBP1 gene, and enhanced dissociation of NCoR, about as well as the full agonist T09. Because both FAS and SREBP1 were evaluated in the same cells, these data strongly suggest that additional *cis*-elements in the SREBP1 gene, perhaps interacting with gene-specific factors, alter the conformation of the GSK224-bound LXR so as to recapitulate that of the full agonist-

bound form. A better understanding of these factors will be critical to the rational design of selective LXR modulators that do not cause hepatic lipogenesis.



Shree Joshi, Joseph Weaver, GSK

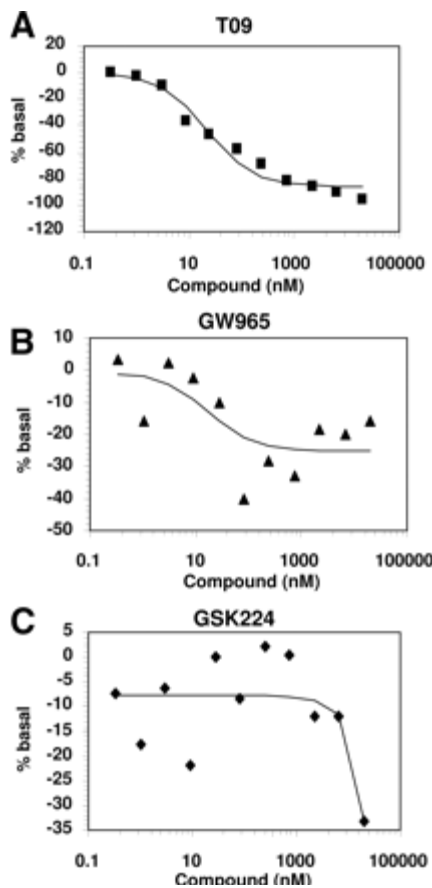
Figure 2.1. Transactivation by LXR Ligands Disproportionate to Coactivator Recruitment. A, Structures of LXR agonists. B, Activity of LXR agonists in transient transfection assay with Gal4-LXR α cotransfected with 5xUAS-SV40-luciferase reporter. EC₅₀ values were 1.0 μ M (GSK224), 2.8 μ M (GW965), and 1.3 μ M (T09). *Inset* shows a magnified view of GSK224 and vehicle data. C, FRET assay for the indicated compounds incubated with LXR α LBD and SRC-1 peptide. EC₅₀ values were 5.90 μ M (GSK224), 6.60 μ M (GW965), and 7.10 μ M (T09). Data shown are representative results (n = 3), and the experiment was repeated two times with similar results.

Table 2.1 LXR Ligand Properties

Table 1. LXR Ligand Properties							
Ligand	Agonism	NCoR Dismissal (FRET)	Gal-LXR α	siNCoR	NCoR O/E	hABCA1-Luc	Gene Expression
GSK224	Weak partial	-7%	↑	5.2 ↑	1.3 ↓	↑	↑ ↑ ABCA1 ↑ ↑ FAS ↑ ↑ SREBP1
GW965	Partial	-23%	↑ ↑ ↑	3.7 ↑	1.8 ↓	↑ ↑	↑ ↑ ABCA1 ↑ ↑ FAS ↑ ↑ SREBP1
T091317	Full	-94%	↑ ↑ ↑ ↑ ↑	2.0 ↑	1.2 ↓	↑ ↑ ↑	↑ ↑ ABCA1 ↑ ↑ ↑ FAS ↑ ↑ SREBP1

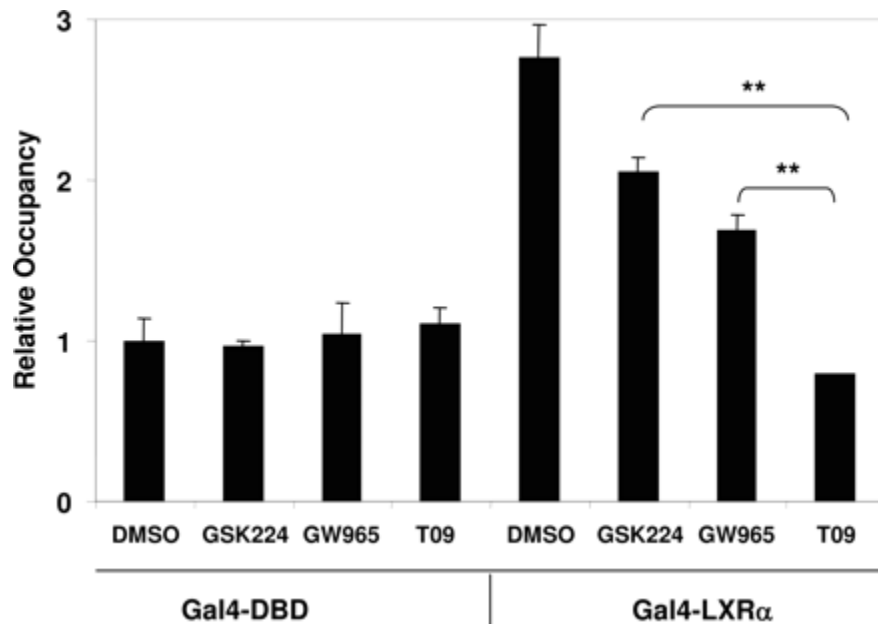
NCoR O/E, Overexpression of NCoR.

Shree Joshi, Joseph Weaver, GSK



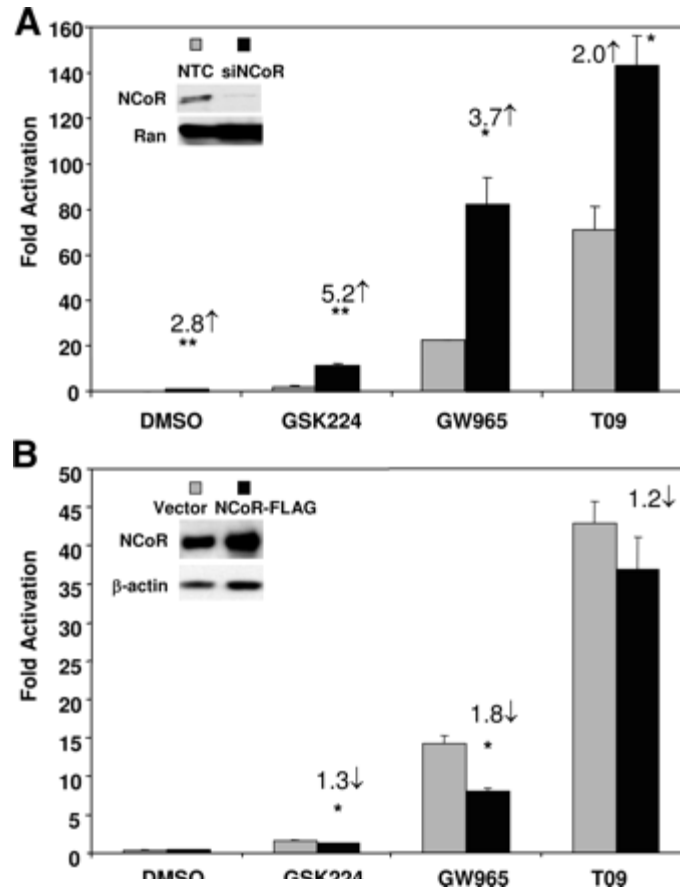
GSK

Figure 2.2 LXR Ligands Differentially Dismiss Corepressor CoRNR Peptide *in Vitro*. Relative FRET for the indicated compounds was determined by incubation of LXR α LBD with NCoR peptide in the presence of increasing ligand concentration. A, T0901317 B, GW965. C, GSK224. EC₅₀ values were 5.01 μ M (GSK224), 7.51 μ M (GW965), and 7.53 μ M (T09). Data shown are representative results, and the experiment was repeated two times with similar results.



Dave Steger

Figure 2.3 Differential Dismissal of Corepressor from LXR on an LXR α -Responsive Reporter Gene 293T cells were cotransfected with 5xUAS-SV40-luciferase and Gal-LXR α or Gal-DBD control and treated with ligand (10 μ M) or vehicle overnight. ChIP assay was performed with anti-NCoR antibody as described in *Materials and Methods*. ChIP signal was normalized to nonspecific 36B4 gene. Gal-DBD was a negative control for NCoR recruitment by ChIP, and relative recruitment was compared with Gal-DBD, DMSO treatment. *Error bars* represent SEM, n = 3 from three independent experiments. **, $P < 0.01$ calculated by Student's t test.



Shree Joshi, Joseph Weaver

Figure 2.4 Modulation of NCoR Levels Differentially Influences Ligand-Dependent Transactivation of LXR α A, NCoR knockdown. 293T cells were cotransfected with 5xUAS-SV40-luciferase and Gal-LXR α along with siRNA against human NCoR or nontargeting control (NTC). Cells were treated with ligands (10 μ M) or vehicle overnight. Relative activation was determined as fold change vs. Gal-DBD controls. *, $P < 0.05$ or **, $P < 0.01$ compared with NTC. NCoR knockdown was confirmed by immunoblotting (*inset*), and Ran protein level is shown as loading control. B, NCoR overexpression. 293T cells were cotransfected with 5xUAS-SV40-luciferase and Gal-LXR α along with human NCoR expression vector or empty control plasmid. Cells were treated with ligands (10 μ M) or vehicle overnight. Each *bar* is the mean value \pm SE (n = 3) from three independent experiments. NCoR expression was confirmed by immunoblotting (*inset*), and β -actin protein level is shown as a loading control. *, $P < 0.05$ compared with vector control for each ligand treatment calculated by Student's *t* test. siNCoR, small interfering NCoR.

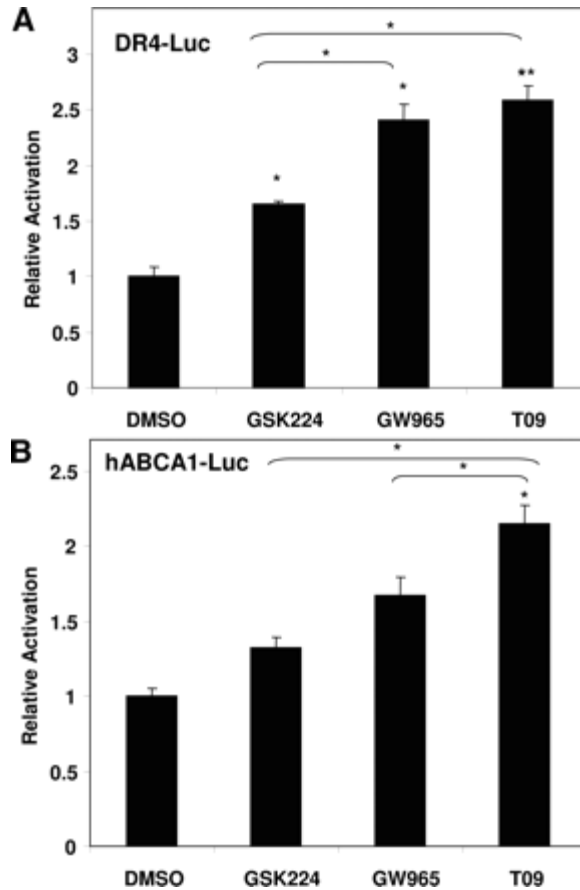


Figure 2.5 Differential Activation of Full-Length LXR. A, DR-4 Element. 293T cells were cotransfected with pCIS control plasmid or 3x-(DR-4)-tk-luciferase, pCMX-LXR α , pCMX-RXR α and β -galactosidase and treated with 10 μ M ligand or vehicle for 18 h. B, hABCA1 promoter activity. 293T cells were cotransfected with pGL3 control plasmid or pGL3 containing a 1.45-kb proximal promoter fragment of the human Abca1 promoter spanning the LXRE site, pCMX-LXR α , pCMX-RXR α , and β -galactosidase and treated with 10 μ M ligand or vehicle for 18 h. Luciferase activity was measured and normalized to cotransfected β -galactosidase activity. Relative activation was determined by normalizing to control vector treated with corresponding vehicle or ligand. Results are representative of a minimum of three independent experiments. Activation upon treatment with DMSO was set to equal 1. Each bar is the mean value \pm SE of triplicate samples from three independent experiments. Statistically significant induction compared with DMSO: *, $P < 0.5$ or **, $P < 0.01$ by Student's t test or between ligands where indicated.

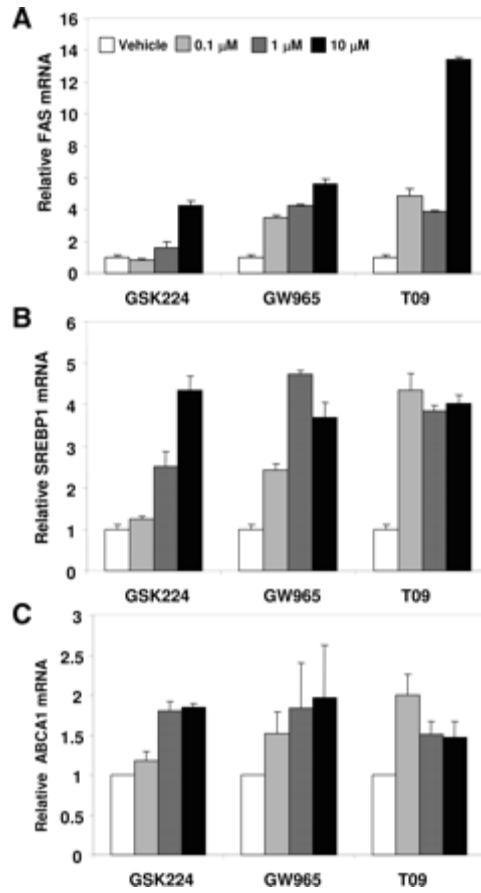


Figure 2.6 Endogenous Gene Regulation by LXR Ligands in Hepatoma Cells

HepG2 cells were seeded in 24-well plates and incubated with increasing concentrations of ligand or vehicle in media containing 10% delipidated media overnight, after which target gene expression was analyzed by quantitative real-time PCR. A, FAS gene expression. B, SREBP1 gene expression. C, ABCA1 gene expression. Results indicate mean \pm SE from triplicates of a representative experiment repeated three times with similar results.

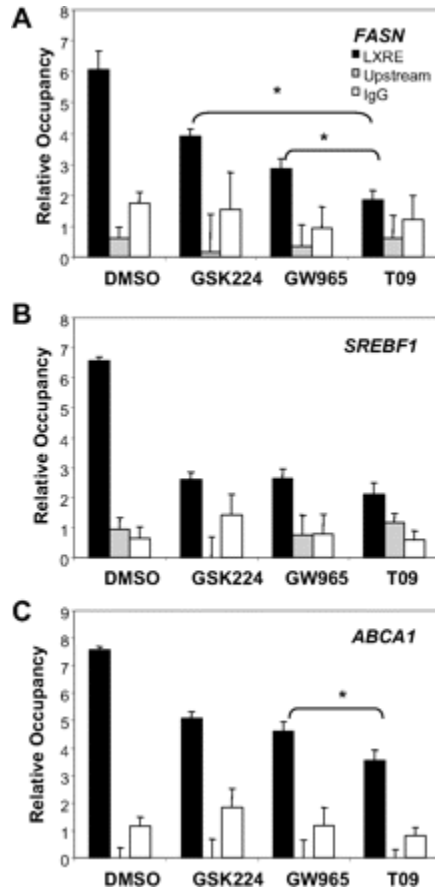


Figure 2.7 LXR Ligands Differentially Recruit NCoR to Endogenous Target Genes
HepG2 cells were seeded in 24-well plates and incubated with ligand (10 μM) or vehicle in media containing 10% delipidated media overnight, after which ChIP analysis for NCoR was performed. A, *FASN* gene. B, *SREBP1c* gene. C, *ABCA1* gene. ChIP signal was normalized to nonspecific DNA region spanning the 36B4 gene, and data represent mean \pm SEM (n = 3). Data are from a representative experiment repeated three times with similar results. *, $P < 0.05$ calculated by Student's *t* test.

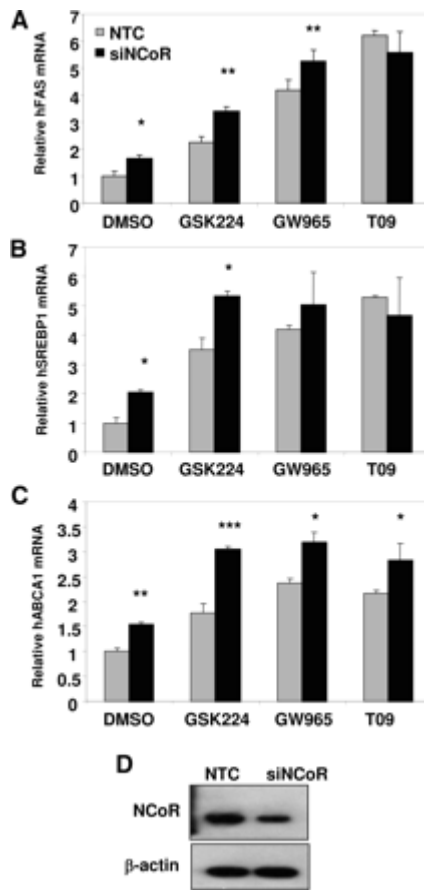


Figure 2.8 NCoR Depletion Induces Basal Activation of LXR Target Genes and Differentially Potentiates Ligand-Dependent Gene Activation HepG2 cells were electroporated with siRNA targeting human NCoR or NTC. Cells were treated with the indicated ligands (10 μ M) or vehicle control overnight in media containing 10% delipidated serum. Relative expression was calculated by normalizing real-time PCR data to 36B4 expression, and fold change indicates relative increase over NTC vehicle-treated cells. A, Fatty acid synthase gene expression. B, SREBP1c gene expression. C, ABCA1 gene expression. Data represent mean \pm SEM of triplicate samples. Data are from a representative experiment repeated three times with similar results. *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.005$ relative to siNTC calculated by Student's t test. NTC, Nontargeting control; siNCoR, small interfering NCoR; siNTC, small interfering NTC.

CHAPTER THREE

STRUCTURAL ANALYSIS OF REV-ERB α REPRESSION BY THE NUCLEAR COREPRESSOR NCoR

Introduction

Nuclear receptors (NRs) comprise a large family of ligand-sensitive transcription factors that regulate growth, development, metabolism, circadian rhythm, and other physiological processes (Desvergne et al., 2006; Mangelsdorf et al., 1995; Teboul et al., 2008). These receptors bind coactivator molecules in the presence of ligand and corepressors in the absence of ligand, which facilitates local chromatin remodeling to effect target gene transcriptional activation or repression (Aranda and Pascual, 2001; Glass and Rosenfeld, 2000; Hu and Lazar, 2000; Privalsky, 2004). NRs contain two modules that contribute to their activation functions. A ligand-insensitive activation domain known as Activation Function-1 (AF-1) is located in the NR N-terminus, whereas the ligand-dependent activation domain, known as AF-2, is contained within the C-terminal ligand binding domain (LBD) (Moras and Gronemeyer, 1998).

Coactivator molecules contain multiple motifs that are sufficient to mediate association with the liganded NR LBD (Darimont et al., 1998; McInerney et al., 1998; Westin et al., 1998; Wurtz et al., 1996). These motifs include the sequence LxxLL (where x is any amino acid and L is leucine), and have been referred to as NR boxes (Heery et al., 1997). Crystal structural studies have revealed that NR LBDs consist of 12 α -helical regions, of which helices 3, 4, 5, and 12 form the surface that binds the NR box (Bain et al., 2007; Moras and Gronemeyer, 1998). Biochemical studies show that helix 12 (H12) is required for transcriptional activation as well as coactivator binding by NRs (Danielian et al., 1992; Feng et al., 1998). Conversely, in the absence of ligand, receptor LBDs associate

with co-repressors such as NCoR (Nuclear Co-Repressor) and SMRT (Silencing Mediator of Retinoid and Thyroid Receptors), which each contain two or three CoRNR (Co-repressor Nuclear Receptor) motifs with a core IxxI/VI sequence (Hu and Lazar, 1999; Hu et al., 2001; Perissi et al., 1999) (Fig. 3.1). CoRNR peptides interact with a surface of the NRs that contains H3, 4, and 5 and thereby overlaps with the binding surface for NR box peptides (Hu et al., 2001; Perissi et al., 1999). However, H12 is not required and, indeed, hinders for the CoRNR interaction with NRs (Chen and Evans, 1995). This is not only of biochemical interest, as it explains the dominant negative activity of the oncogene v-erbA (Saaticioglu et al., 1993) as well as naturally occurring thyroid hormone receptor mutations that cause the clinical syndrome of resistance to thyroid hormone (Chatterjee et al., 1991; Yoh et al., 1997; Yoh and Privalsky, 2000).

The structural determinants of nuclear hormone receptor-mediated repression were first demonstrated by X-ray crystallography of an antagonist-bound PPAR α co-crystallized with a CoRNR peptide derived from SMRT (Xu et al., 2002). Interestingly, this structure revealed that the NR interacts not only with the core IxxI/VI CoRNR motif but also with an extended α -helix in the CoRNR box. Although its I/LxxI/VI core led to the naming of the CoRNR box by analogy to the coactivator NR box "LXXLL" (Hu and Lazar, 1999; Nagy et al., 1999a), this critical detail was more consistent with earlier suggestions that the key interaction motif consists of the sequence LxxHIxxxI, with the first 4 amino acids (LxxH) comprising an extended helix and the last 5 amino acids (IxxxI) corresponding to the I/LxxI/VI CoRNR motif (Perissi et al., 1999; Xu et al., 2002). The key difference is

that, in the extended helix model and in the PPAR α /SMRT crystal structure, the fourth amino acid of the CoRNR motif does not contribute to the interaction. However, Nagy et al showed that mutation of "ISEVI" to "ISEAI" in a SMRT CoRNR peptide, which destroys the "IxxV/II" motif but does not affect the "LXXXIXXX^L/I" motif, abrogates corepressor interaction with thyroid hormone receptor (TR) (Nagy et al., 1999b). Thus, it is unclear whether binding of corepressors to NR requires the IxxII, or "two-turn" CoRNR box motif or the extended "LXXXIXXX^L/I", or the "three-turn" CoRNR box motif (Figure 3.1).

Of particular interest to repression by NRs is Rev-erb α , a receptor for heme (Raguram et al., 2007; Yin et al., 2007), that lacks H12 and thus does not contain an AF-2 domain (Lazar et al., 1989; Miyajima et al., 1989). Rev-erb α exerts its repressive activity by association with NCoR (Harding and Lazar, 1995; Yin and Lazar, 2005; Zamir et al., 1997a), and has a preference for the CoRNR motif contained in NCoR amino acids 2024-2065 which has been variously referred to as ID1 (Hu et al., 2001) or ID2 (Perissi et al., 1999). NCoR binding to Rev-erb α has important biological consequences including repression of the circadian clock transcription factor Bmal1 to influence the period length and circadian amplitude (Preitner et al., 2002; Yin and Lazar, 2005). Additionally, Rev-erb α also directly regulates genes and metabolic processes including the adipokine PAI-1 (Wang et al., 2006), the gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Yin et al., 2007), lipid metabolism genes including Apo-AI and ApoC-III (Coste and Rodríguez, 2002; Coste and

Rodriguez, 2002; Raspe et al., 2002), hepatic cholesterol and bile acid metabolism (Duez et al., 2008; Le Martelot et al., 2009), muscle differentiation (Downes et al., 1995), adipocyte differentiation (Wang and Lazar, 2008) and the transcriptional coactivator PGC1 α (Wu et al., 2009). Thus, a molecular understanding of its repression function is critical for further study of the basis for nuclear hormone receptor-mediated repression as well as to provide insight into the potential pharmacological manipulation of this receptor in disease.

Here we present the crystal structure of the Rev-erb α LBD co-crystallized with an NCoR-derived CoRNR peptide. We demonstrate using alanine substitution that this receptor utilizes the core IxxII “two-turn” CoRNR box motif to facilitate corepressor interaction, and thus this represents a different mode of binding compared to that of the PPAR α /SMRT extended “three-turn” α -helical CoRNR box motif interaction. Furthermore, performing alanine substitutions of key hydrophobic residues within the LBD predicted to make important contacts with the CoRNR box demonstrates the necessity of these residues for Rev-erb α /NCoR-mediated repression. Thus, this structure provides insight into the nature of the repressive function of Rev-erb α and, more generally, demonstrates that different NRs may utilize different CoRNR motif registries.

Experimental Procedures

Plasmids. Human FLAG-Rev-erb α , VP16-Rev-erb α , Gal-DBD, 5xUAS-SV40-Luciferase, DR-2-Luciferase, Bmal1-Luciferase and Gal-NCOR-ID1 have been described previously (Harding and Lazar, 1995; Yin and Lazar, 2005; Zamir et al., 1997b). Site-directed mutagenesis was performed using Stratagene site-directed mutagenesis II XL according to manufacturer's instructions and commercially available primer design program (Stratagene). All point mutants were verified by sequencing.

Cell Culture and Transfection. 293T cells were maintained in DMEM plus 10% fetal bovine serum in .5% penicillin/streptomycin at 37°C, 5% CO₂. 24 hours prior to transfection, cells were seeded into 24-well plates. Transfections were performed in fresh media lacking antibiotics. For mammalian two-hybrid assays, each well was transfected with 0.25 μ g Gal-ID1 of Gal-DBD, 0.25 μ g of VP16 or VP16-Rev-erb α/β , 0.25 μ g of 5xUAS-SV40-Luciferase, and 0.1 μ g of β -galactosidase and 2 μ l/well of Lipofectamine 2000 (Invitrogen) in 100 μ l Opti-MEM. For repression assays, cells were co-transfected with 0.2 μ g DR-Luciferase or Bmal1-Luciferase, 0.6 μ g of pcDNA or FLAG-Rev-erb α and 0.1 μ g of β -galactosidase. For gene expression analysis, cells were co-transfected with 0.6 μ g plasmid. Transfections were carried out for 36 hours and cells lysed in Passive Lysis Buffer (Promega) or Buffer RLT (Qiagen) for mRNA extraction.

Luciferase Assay. Cells were lysed in 100 μ l of Passive Lysis Buffer (Promega) followed by freeze-thaw at -80° C. 5 μ l of lysate was used for luciferase assay (Promega) or β -galactosidase assay. Relative light units for luciferase were normalized to β -gal activity.

Gene Expression Assay. Lysates were processed using RNeasy (Qiagen) and mRNA quantitated. Reverse transcription was performed using the High Capacity cDNA Synthesis Kit (Applied Biosystems). 20ng of cDNA was amplified using primers for human *Bmal1* (Qiagen) and *Gapdh* (Qiagen) using SYBR Green Quantitative PCR Master Mix (Applied Biosystems) on a Real-Time 7700 (Applied Biosystems). Data was normalized to *Gapdh* housekeeping gene.

Immunoblotting. Extracts were electrophoresed by SDS-PAGE on a 4-20% Tris-Glycine gradient gel (Invitrogen). Protein transfer was carried out on PVDF Immobilon-P membranes (Amersham) overnight at 22V at 4° C. Membranes were blocked for 90 minutes in Tris-buffered saline/0.1% Tween-20 (TBST) containing 5% non-fat milk with gentle agitation at room temperature. Membranes were incubated in blocking buffer containing primary antibodies overnight at 4° C. The following antibodies were used: rabbit anti-NCoR (REF), mouse ant-FLAG (Sigma-Aldrich) and rabbit anti-HDAC3 (Upstate). Following four washes in TBST, secondary antibodies (Pierce) were applied

for one hour at room temperature. Following four TBST washes, membranes were processed using ECL Plus (Amersham).

Results

Crystallization of Rev-erb α LBD bound to NCoR peptide. To further characterize the repression function of Rev-erb α , a soluble deletion mutant of the human Rev-erb α LBD was constituted and co-crystallized with the highest affinity NCoR-containing CoRNR peptide (amino acids 2045-2065) and the structure derived to 2.6Å. Examination of the LBD structure revealed a canonical 3-layer α -helical sandwich comparable to other published NR LBD structures (Nettles and Greene, 2005) (Fig 3.2B). Remarkably, given that the Rev-erb α LBD lacks H12, we observed a short anti-parallel β -sheet derived from the extreme C-terminus of the LBD as well as the N-terminus of the CoRNR peptide. Following this β -strand, the NCoR peptide adopts an amphipathic α -helical conformation that contacts regions of the ligand-binding pocket, specifically helices 3, 4, and 5 and terminates at a conserved H3 residue, K455. However, this internally deleted clone produced regions devoid of electron density for residues between H1 and H3 and the generally conserved intra-molecular β -sheet region between H5 and H6, as illustrated by the red arrows.

Two regions of Rev-erb α underlie its interaction with NCoR. The Rev-erb α LBD contains several regions that have been previously described to be important for its

repression function, including helix 1, domain within the poly-proline region, helix 3, and C-terminal domain encompassing amino acids 602-614 (Fig.3.2A) (Renaud et al., 2000). The unique anti-parallel β -strand between the C-terminus of Rev-erb α (amino acids 609-614) and the N-terminus of the CoRNR box peptide (2047-2049) corresponds to a predicted interaction region in the C-terminus that is required for Rev-erb α repression (Renaud et al., 2000). Rev-erb α residue Trp⁴³⁶ underpins and supports the previously described Y-domain β -strand. Phe⁶⁰⁹ provides packing interactions near Ile²⁰⁴⁹ of NCoR ID1. N-CoR Leu²⁰⁵¹ marks the start of an amphipathic α -helix (below left) that directs hydrophobic residues Leu²⁰⁵¹, Ile²⁰⁵⁵, Ile²⁰⁵⁸, Ile²⁰⁵⁹ and Phe²⁰⁶³ towards the mostly hydrophobic Rev-erb α binding surface (Fig 3.3A). Noted hydrogen bonds are Thr⁴⁴⁴ to His²⁰⁵⁴ and Lys⁴⁷³ to Thr²⁰⁶⁰ of NCoR and a water-mediated interaction from the conserved charge clamp Lys⁴⁵⁵ to the Asp²⁰⁶² ID1 backbone.

Structure-guided analysis of repression by full length Rev-erb α . As the structural analysis predicted that Rev-erb α contains several hydrophobic residues that make important contacts with the amphipathic α -helical CoRNR motif, we next used alanine substitution mutagenesis to probe the contribution of specific residues to the repression function of full length Rev-erb α in cells. As expected, full-length human Rev-erb α potently represses a reporter gene containing a direct repeat of ATTGCA nucleotides spaced by two bases (DR-2), which is the canonical Rev-erb α DNA binding sequence (Harding and Lazar, 1995) (Fig. 3.3A). However, mutation of Trp⁴³⁶, Phe⁴⁴³, Val⁴⁴⁷, Val⁴⁵¹, Arg⁴⁶¹, Leu⁴⁷², Phe⁴⁷⁷, as well as several residues at the extreme C-terminus

including Leu⁶⁰⁶, Phe⁶⁰⁹, and Arg⁶¹⁰, resulted in a marked loss of repression function (Fig. 3.5a). As a control, mutation of Gln⁴³³, which was not predicted to contact the NCoR peptide, had minimal effects on repression function. Interestingly, point mutations of Trp⁴³⁶, Phe⁶⁰⁹ and Arg⁶¹⁰, which underpin the anti-parallel β strand shown to be required for repression (Fig. 3.4A), minimally affected repression, suggesting that the single amino acid substitutions are insufficient to completely disrupt interaction. In addition to the reporter gene assay, the Phe⁴⁴³, Val⁴⁴⁷, and Phe⁴⁷⁷ mutants were all defective in repressing expression of the endogenous Bmal1 gene (Fig. 3.5B). Together, these data suggest that these residues mediate important contacts with the NCoR CoRNR box motif and are required for Rev-erb α repression of Bmal1.

Novel mode of binding of Rev-erb α to the NCoR CoRNR motif. Superimposition of the NCoR CoRNR peptide (magenta) bound to Rev-erb α with PPAR α bound to the SMRT CoRNR peptide (white) revealed different binding modes for the corepressor peptides (Fig. 3.6A, 3.6B). Most notably, the extended α -helix of the NCoR peptide ("LADH", defined as residues i+1 to i+4) do not make significant contacts with Rev-erb α , whereas the isoleucine residues in the core "two-turn" CoRNR box motif "ICQII" are critical. This difference equates to a 4 position N-terminal shift in the NCoR CoRNR peptide alignment bound to Rev-erb α relative to the binding of SMRT CoRNR peptide to PPAR α . The formation of the β -sheet structure between NCoR and Rev-erb α is likely to account for the shift. Interactions such as hydrogen-bonding between Lys⁴⁷³ to Thr²⁰⁶⁰, a unique threonine in the NCoR peptide, potentially contribute to motif selection as well.

Next, we tested this novel mode of CoRNR peptide binding predicted by the structure functionally. The effects of mutation in the NCoR CoRNR peptide on binding to the Rev-erb α LBD were determined *in vitro* by fluorescence resonance energy transfer assay (Fig. 3.7A). As hypothesized from the structure, mutation of residues i+1 and i+4 had minimal effects on binding affinity. By contrast, mutation of residues i+5, i+8, i+9, which comprise the core CoRNR motif, dramatically reduced binding affinity for Rev-erb α LBD.

The structural predictions were also tested in living cells using a mammalian two-hybrid assay. Co-transfection of the NCoR-CoRNR peptide fused to the DBD of the Gal4 transcription factor, with Rev-erb α fused to the VP16 transcriptional activation domain led to the potent activation of a luciferase reporter gene containing Gal4 binding sites, indicative of an interaction between the NCoR peptide and Rev-erb α (Fig. 3.7B). Mutations in the CoRNR residues i+5, i+8, and i+9 abolished the interaction, whereas those in the extended N-terminal α -helix (i+1, i+4) had little effect. Thus, the core "IxxII" motif was required for interaction with Rev-erb α , whereas the "LADH" extension was not.

The novel mode of CoRNR binding extends to the TR. To further explore this novel mode of CoRNR binding to NRs, we tested chimeric CoRNR-derived peptides constructs

for their ability to interact with thyroid receptor (TR), which also preferentially binds NCoR(Ishizuka and Lazar, 2003). In ID1 and ID2 of N-CoR, these sequences are "ICQII" and "LEDII", respectively (Fig. 3.7a). In the alignment of based on the antagonized PPAR α :SMRT crystal structure and previous reports, ID2 is shifted relative to ID1 by 4 amino acids toward the N-terminus, such that the "ICQII" of the first interaction domain is aligned with "IRKAL" (Fig. 3.7A) (Perissi et al., 1999; Xu et al., 2002). Indeed, in the co-crystal structure of antagonist bound PPAR α and a peptide derived from the second interaction domain of SMRT, the L of the "IRKAL" motif, which is downstream of the putative CoRNR box "LEDII", makes important contacts with PPAR α . This is not inconsistent with the CoRNR box alignment, because we and others have shown that the core motif is necessary but not sufficient; specific flanking amino acids are also required, presumably due to important contacts with the receptor such as those demonstrated in the structure.

We tested the two potential alignments by making hybrids of ID1 and ID2, with ID2 sequences following the "ICQII" of the ID1 domain (ID1/ID2) or replacing the "ICQII" with "IRKAL" (ID1/ID2-RKAL)(Fig 3.7B). Both hybrid peptides contain the extended "LXXXIXXXL/I" motif, however, while the peptide with the LEDII motif substitution was able to interact with TR, the peptide with the IRKAL motif (but lacking any "I/L X X I/V I") did not (Fig. 3.7B). These data, together with those of Nagy et al showing that mutating the CoRNR motif but not the "LXXXIXXXL/I" abrogates corepressor interaction with TR (Nagy et al., 1999b), suggest that TR interacts with NCoR by in a

manner similar to that of Rev-erb α , involving interaction with the core “I/L X X I/V I” CoRNR motif.

Discussion

Nuclear hormone receptors can actively repress transcription via recruitment of the corepressors NCoR and SMRT. NCoR and SMRT binding is mediated by C-terminal interaction domains, which contain α -helical isoleucine rich motifs, termed CoRNR box motifs which are structurally analogous to the motif which drives coactivator binding. The original reports of these CoRNR motifs varied in their requirement of an additional N-terminal α -helical region. The “two-turn” CoRNR motif is simply IxxII whereas the “three-turn” CoRNR motif is LxxHIxxxI.

Since the binding modes of coactivators and corepressors are virtually identical and the regions of the NR ligand-binding domains they contact are overlapping, efforts were made to address the relative selectivity of coactivator versus corepressor binding structurally. Taking advantage of the known structure activity relationship of a potent synthetic ligand for PPAR α , a novel antagonist was generated that impaired PPAR α activity and enhanced binding to SMRT (Xu et al., 2002). X-ray crystal structure of the ternary PPAR α /antagonist/SMRT complex revealed that SMRT adopted a three-turn α -helical structure. Interestingly, the extended “three-turn” CoRNR motif adopted in this structure by SMRT resulted in the repositioning of helix 12, which is critical for

coactivator binding, and thus provided a molecular mechanism for antagonist-mediated PPAR α /SMRT interaction.

To further understand the structural basis for physiological repression by nuclear hormone receptors we co-crystallized a binary complex of NCoR and the ligand-binding domain for Rev-erb α , which does not contain helix 12 and thus functions as a constitutive receptor. This structure immediately revealed a fundamental difference in the mode of PPAR α /SMRT and Rev-erb α /NCoR binding. The NCoR CoRNR motif was N-terminally shifted in its mode of binding, utilizing a “two-turn” CoRNR motif as opposed to the “three-turn” utilized by SMRT. Furthermore, the N-terminus of the CoRNR motif adopted an anti-parallel β -sheet motif with the extreme C-terminus of Rev-erb α . To ask whether this CoRNR motif binding mode could be recapitulated in vivo, we mutated residues predicted to be critical for Rev-erb α NCoR binding and repression. Indeed, these results confirmed the “two-turn” CoRNR motif mode is physiologically required for repression by full-length Rev-erb α in cells, whereas mutations of residues predicted for a “three turn” interaction surface were not. These results suggest that nuclear hormone receptors can utilize differing modes of co-repressor binding depending upon the receptor/corepressor interaction.

Recently, several X-ray structures of the highly related receptor Rev-erb β have been solved and reported (Pardee et al., 2009b; Woo et al., 2007). One structure reflects apo-

Rev-erb β whereas the other structure reports the heme-bound Rev-erb β receptor. During this study it was discovered that indeed, Rev-erb α has a putative ligand, heme, and that binding of the ligand stabilized the receptor binding with its corepressor NCoR. Binding of the ligand was shown to be dependent on a critical histidine in the C-terminus of the ligand-binding domain, His⁶⁰². Efforts were made to co-crystallize a tertiary complex of Rev-erb α -LBD:NCoR-ID1:heme, but were ultimately unsuccessful. Interestingly, the report of Rev-erb β :heme crystal structure remarkably showed coordination of the analogous histidine, His⁵⁶⁸ as well as contacts with a cysteine, Cys³⁸⁴ contained within the hinge domain. The deletion mutant construct comprising Rev-erb α LBD used in this study did not contain this cysteine residue, Cys⁴¹⁷, and may explain an inability to co-crystallize the complex. Consistent with this, mutation of the analogous Cysteine, Cys⁴¹⁷ to Alanine significantly diminished the repression function of Rev-erb α , consistent with requirement of other residues to facilitate heme binding (shown). This suggests that while His⁶⁰² is necessary for Rev-erb α :heme binding, it is likely not sufficient. Altogether, the receptor:heme:co-repressor complex appears to be liganded by critical histidine and cysteine residues necessary for heme binding which promote interaction between liganded Rev-erb α and the CoRNR box of NCoR.

An understanding of the structural basis for repression by nuclear hormone receptor repression provides a molecular basis for structure-guided efforts in the development of synthetic ligands and antagonists for Rev-erb α and other nuclear hormone receptors. The physiological significance of Rev-erb α repression has recently been demonstrated in NCoR mutant mice (Alenghat et al., 2008). These mice, which are unable to sufficiently

repress Bmal1, have altered circadian period length and are also resistant to diet-induced obesity. Furthermore, the identification of the Rev-erb ligand heme, which facilitates recruitment of corepressor has also led to the report of a selective, synthetic ligand for Rev-erb receptors (Meng et al., 2008) This ligand was shown to directly affect the circadian clock phase through modulation of Rev-erb repressive activity. Given the role of Rev-erb receptors in regulating the positive limb of the circadian clock as well as regulation of physiological pathways including obesity, cholesterol homeostasis, gluconeogenesis and lipid metabolism, the pharmacological manipulation of Rev-erb activity in the treatment of biomedical disease is becoming increasingly plausible (Alenghat et al., 2008; Coste and Rodriguez, 2002; Duez et al., 2008; Raspe et al., 2002; Yin et al., 2007). Thus, an increased understanding of the structural basis for its repressive function is integral to pursuit of pharmacological targeting of this receptor.

**TWO-TURN
"CoRNR" alignment**

```

                                IxxII
NCoR ID1:  VPRTHRLITLADHICQIITQDFAR
SMRT ID1:  PGVKGHQRVVVTLAQHISEVITQDYTR
NCoR ID2:  GHSFADPASNLGLEDIIRKALMG
SMRT ID2:  QAVQEHASTNMGLEAIIRKALMG
```

**THREE-TURN
"LXXXIXXXI" alignment**

```

                                L
                                LXXXIXXXI
NCoR ID1:  VPRTHRLITLADHICQIITQDFAR
SMRT ID1:  PGVKGHQRVVVTLAQHISEVITQDYTR
NCoR ID2:  GHSFADPASNLGLEDIIRKALMG SFD
SMRT ID2:  QAVQEHASTNLGLEAIIRKALMGKYD
```

Figure 3.1 Model of two-turn vs. three-turn for interaction domains of NCoR and SMRT. TWO-TURN "CoRNR alignment" of NCoR and SMRT, which utilizes an IxxII motif. THREE-TURN α -helical CoRNR box alignment, which utilizes and LxxxIxxxI/L motif and predicted residues in the ID of NCoR and SMRT are shown.

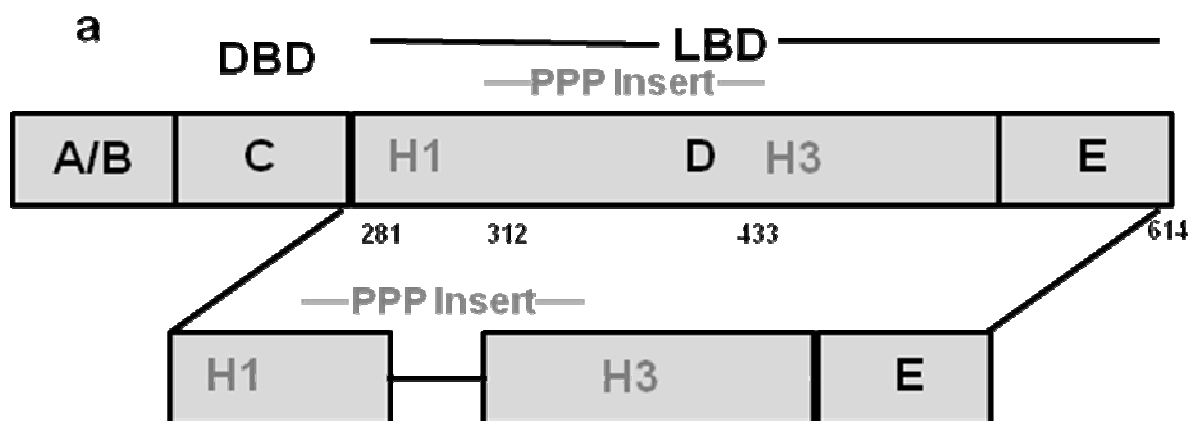


Figure 3.2. Co-crystallization of Rev-erb α Δ 323-423LBD and NCoR Interaction Domain 1 (2040-2065). A). Depiction of functional domains of full-length Rev-erb α . A/B domain, C domain (DBD), D domain (hinge region) and E domain (LBD). Helix 1 and 3 (H1 and H3) and the poly-proline insert region (amino acids 312-433) are noted. The deletion mutant used for crystallization lacks a region of the poly-proline insert, amino acids 323-423.

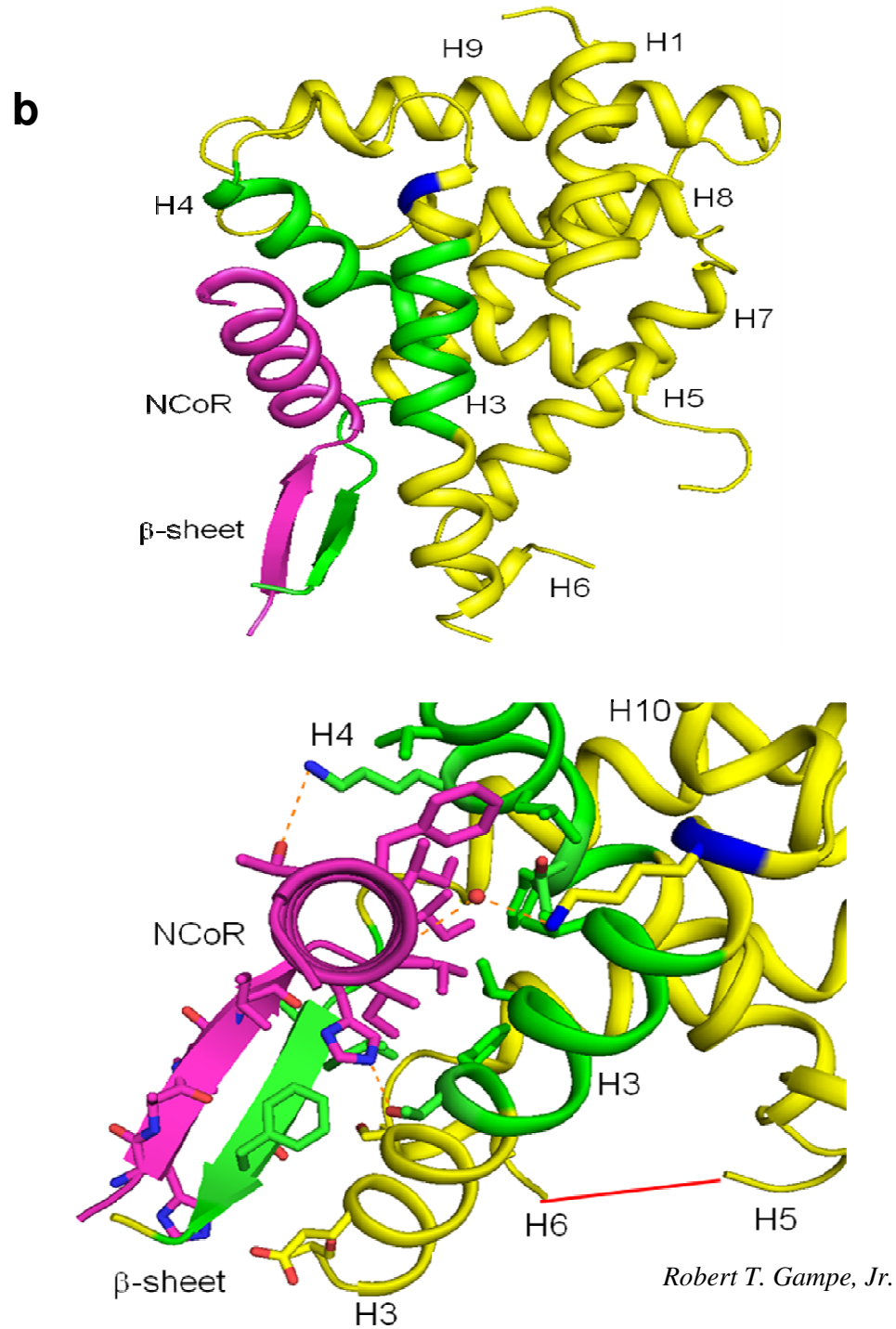
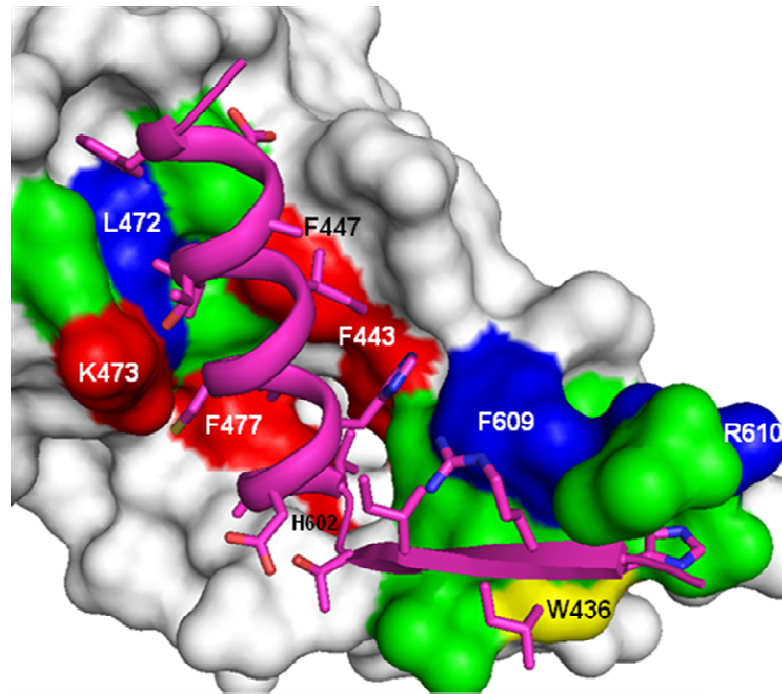
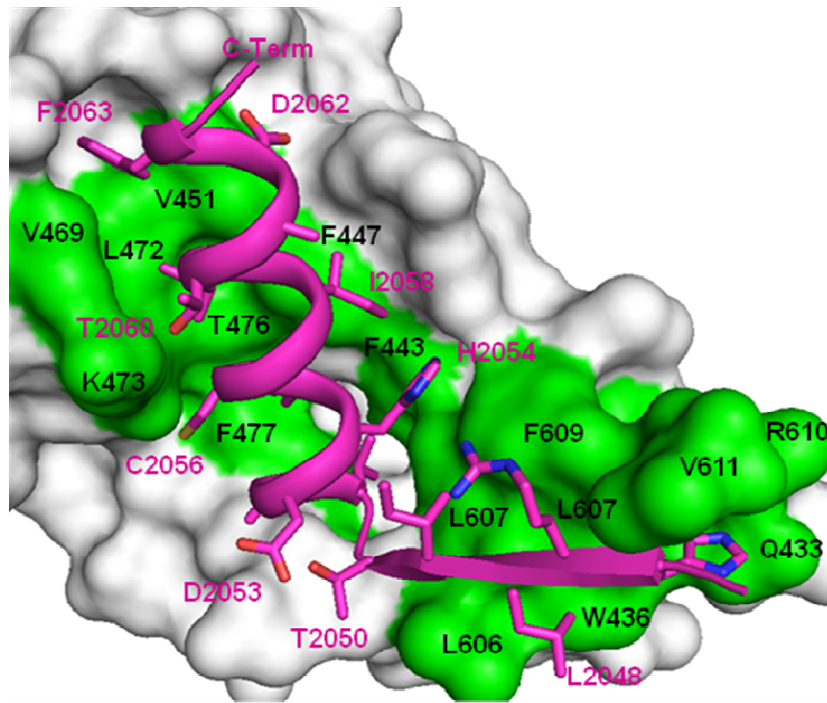


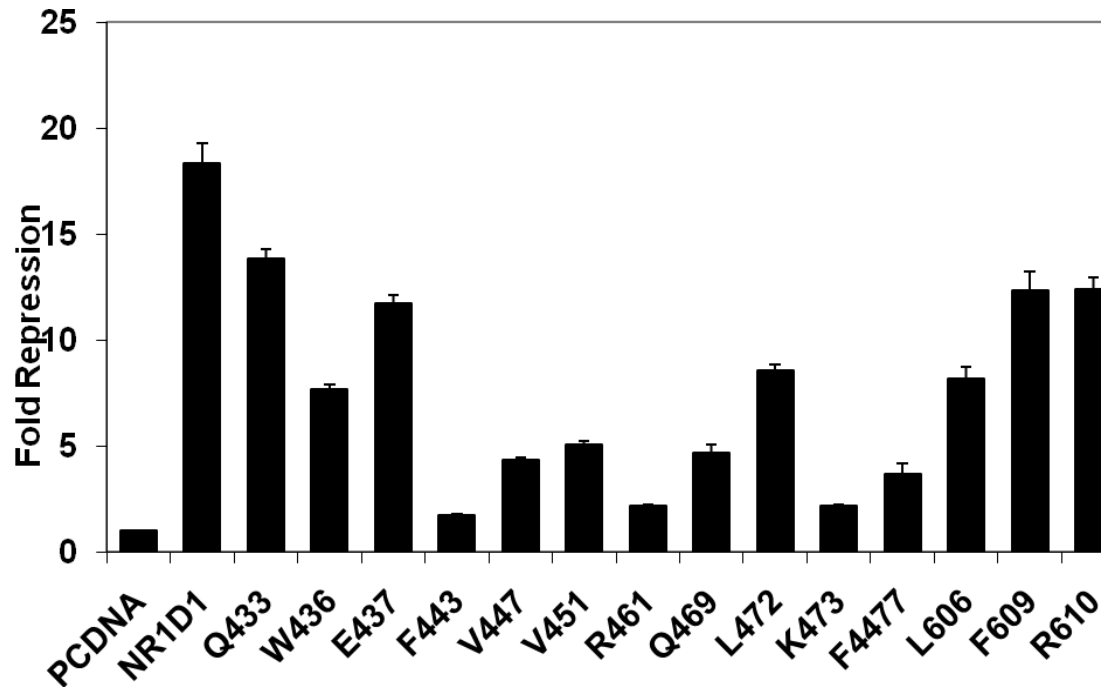
Figure 3.3B Two views of the NCoR Rev-erb α LBD crystal structure with labeled helices. Rev-erb α (281-614 delete 324-422, yellow) mostly conforms to the canonical nuclear receptor LBD α -helical fold and binds the NCoR CoRNR box peptide (magenta) at the AF2 domain (green). Devoid of a classical AF2 helix, the native Rev-erb α C-terminus adopts a short β -strand (green) structure and with the NCoR N-terminus forms an inter-molecular anti-parallel β -sheet. NCoR then switches to an amphipathic α -helical conformation that contacts the classical activation function2 (AF2) region defined by helices 3, 4, and 5 (green) and terminates without directly hydrogen bonding to the conserved charge clamp residue, K455 (Ackerman et al.) in H3. Unstructured regions between H1-H3 and H5-H6 are indicated with red lines.



Robert T. Gampe, Jr.

Figure 3.3 Detailed analyses of key Rev-erb α residues which comprise the contact surface for the NCoR peptide. Residue L2051 of NCoR initiates an amphipathic α -helix comprised of the hydrophobic residues L2051, I2058, I2059, and F2063. Furthermore, examination of the Y-domain, which has previously been shown to be important for Rev-erb α repression, revealed an anti-parallel β -strand formed by residues 2047-2049 of NCoR and 606-609 of Rev-erb α . Hydrogen bonds are indicated.

A



B

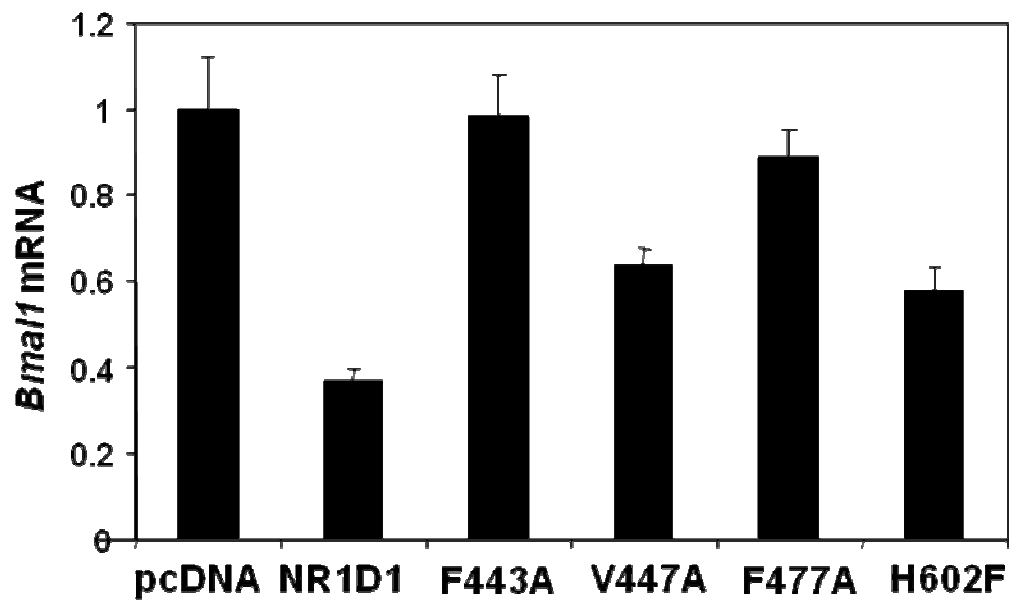


Figure 3.4 Key Hydrophobic Residues of Rev-erb α which mediate NCoR binding are required for Rev-erb α repression function. A). 293T cells were transiently co-transfected with empty vector (control), wild-type Rev-erb α or alanine mutants at indicated residues in addition to a plasmid containing a DR-2 response element upstream of a luciferase reporter. A plasmid containing β -galactosidase was also co-transfected to control for transfection efficiency. At 48 hours, cells were lysed and analyzed for luciferase and β -galactosidase activity. After normalizing to β -gal activity, relative repression was determined by calculating fold reduction compared to empty vector. B). 293T cells were transfected with empty vector, wild-type Rev-erb α , alanine mutants, or the heme-binding H602F mutant. 48-hours post-transfection mRNA was collected and Bmal1 mRNA expression is reported compared to 36B4 housekeeping gene. All data is mean of three independent experiments +/- SEM.

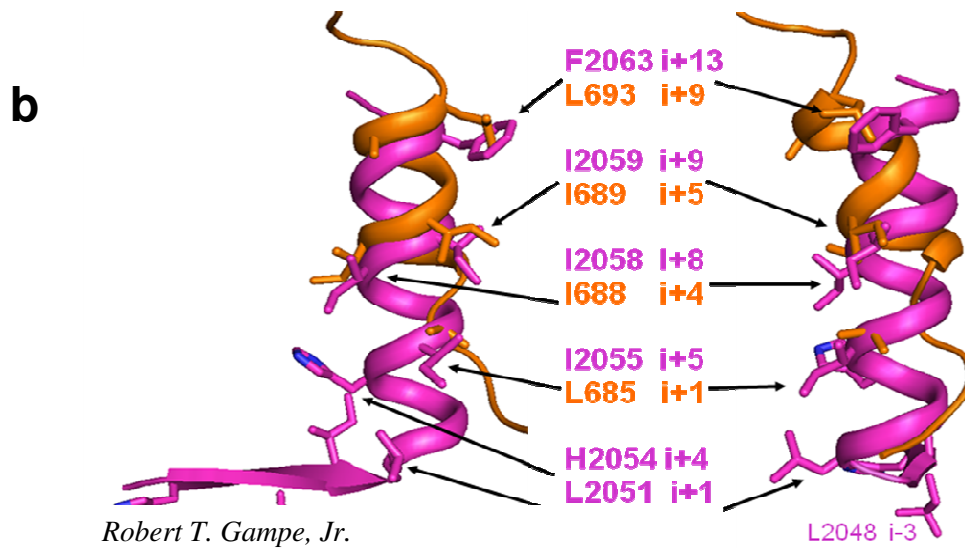
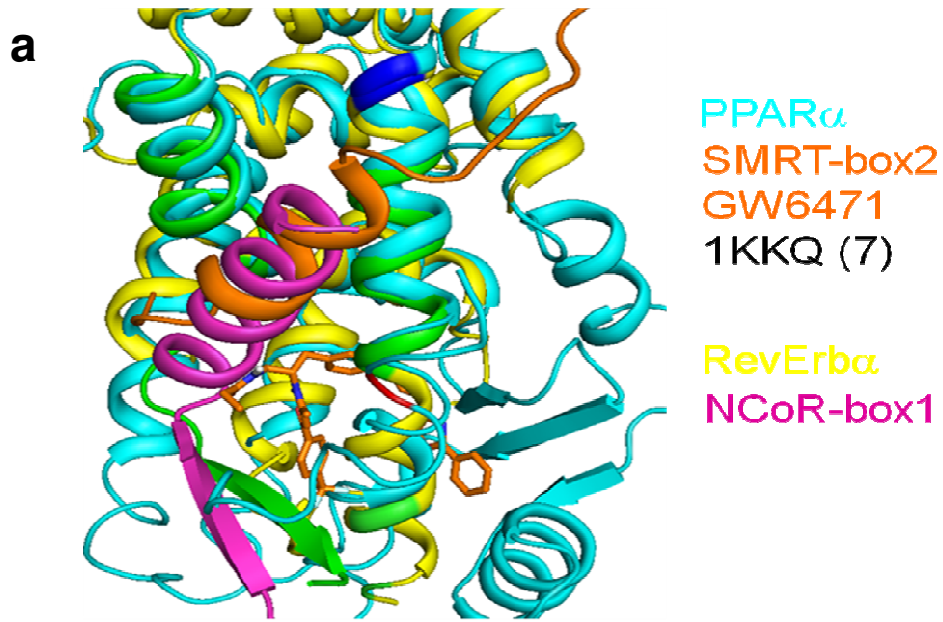


Figure 3.5 NCoR binds Rev-erb α differently than antagonized PPAR α binds SMRT. A) Overlay of NCoR/Rev-erb α (yellow) crystal structure with SMRT/PPAR α (aqua) structure. NCoR is the white ribbon whereas SMRT corepressor is purple. B) Focusing on the modes of corepressor binding, the relative N-terminal shift of NCoR binding compared to that of SMRT bound to PPAR α is depicted.

a**TR-FRET**

NCoR Peptide	Amino Acids	Binding (μM)	Fold Decrease	Contact <5Å
WT	2040-2065	0.22	-	
H2046A	i-5	0.54	2.5	D437, S608
L2048A	i-3	0.57	2.5	W436, L606
L2051A	i+1	0.60	2.7	F443, F447
H2054A	i+4	0.25	1.2	L607, F609
I2055A	i+5	50.3	229.0	V447, F477
I2058A	i+8	8.1	36.8	T441, V447
I2059A	i+9	19.4	88.2	L472, K473
F2063A	I+13	1.9	8.6	V451, V469

Robert T. Gampe, Jr. GSK

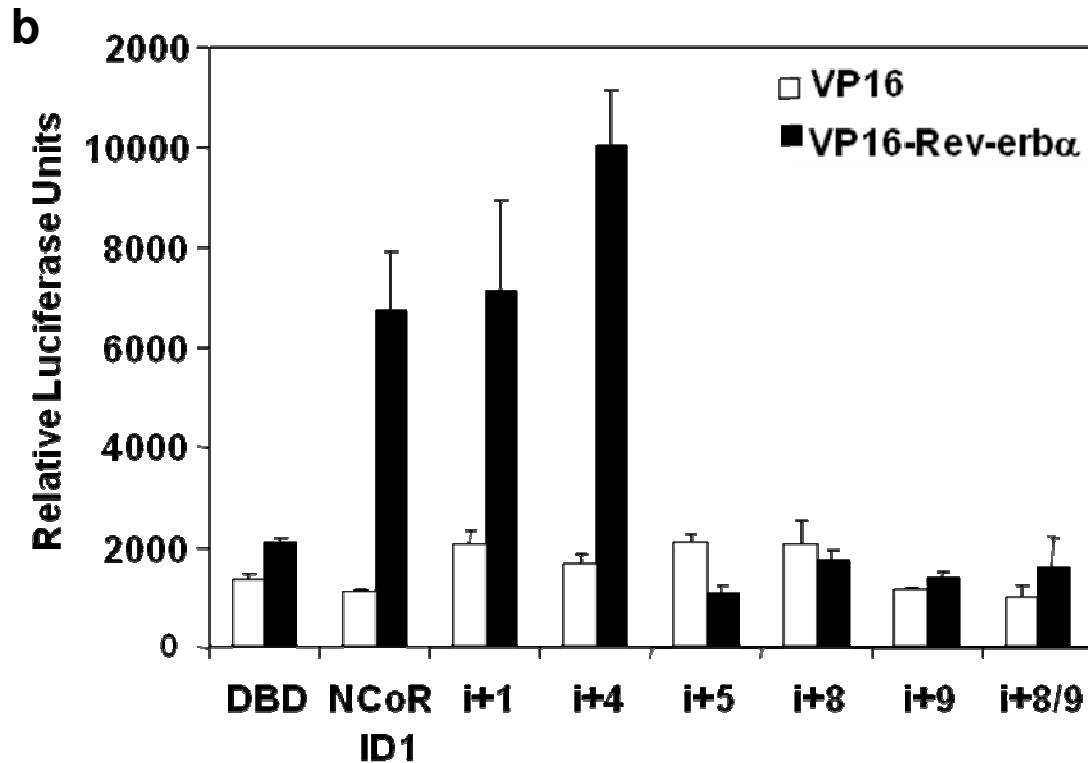


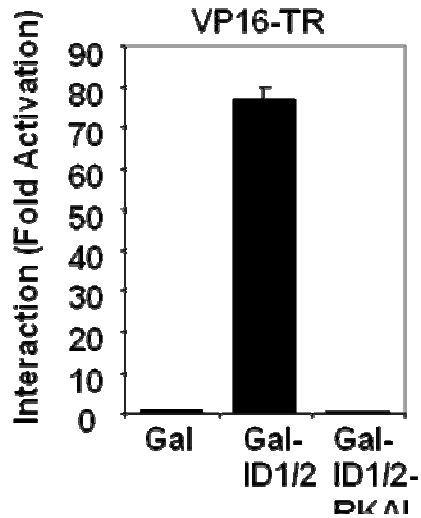
Figure 3.6 Rev-erb α repression requires the IxxII CoRNR box motif of NCoR. A). GST-Rev-erb α LBD was incubated with increasing amounts of wild-type NCoR ID1 peptide or alanine mutants as indicated in a TR-FRET assay. Relative binding affinity of NCoR ID1 as well as mutants was determined. Fold decrease is reported compared to wild-type. Amino acids of Rev-erb α which mediate close molecular contact to the indicated NCoR residues are noted. B). 293T cells were co-transfected with Gal-DBD or Gal-NCoR ID1 wild-type or mutants and either VP16 or VP16 fused to full-length Rev-erb α , as well as a 5xUAS luciferase reporter and β -galactosidase (to measure transfection efficiency). Relative interaction was determined as luciferase units normalized to β -galactosidase activity. All data is mean of three independent experiments +/- SEM.

a

L
LXXXIXXXI

ID1/2: MGQVPRTHRLITLADHICQIIRKALMG^SFD
ID1/2-RKAL: MGQVPRTHRLITLADHIRKALMG^SFD

b



Xiao Hu

Figure 3.7 Nuclear hormone receptors utilize different modes of corepressor binding. A). Gal- ID1/2 construct is shown, which utilizes the two-turn “CoRNR alignment”. Gal ID1/2-RKAL construct is shown, which utilizes the extended α -helical motif sequence as in B. B) Mammalian two-hybrid assay using indicated Gal-fusions co-transfected in 293T cells along with VP16-thyroid receptor (TR) and Gal-5xUAS-luciferase reporter. Following normalization to β -gal control, relative interaction is shown normalized to Gal-DBD.

CHAPTER FOUR

SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

This thesis has explored the molecular mechanisms of NCoR repression in selective modulation of nuclear hormone receptors including LXR as well as the structural basis for physiological repression by Rev-erb α . In chapter two, we explored the relative transcriptional effects of full versus partial agonists on LXR α transcriptional activation. Full agonists such as T09 robustly activate LXR α whereas partial agonists such as GW965 and GSK224 exhibit dramatically lower levels of LXR activation. Furthermore, even amongst partial agonists, there is a relative spectrum of activity, such that GSK224 can be considered a very weak agonist as its activity is far reduced compared to the intermediate partial agonist GW965. We hypothesized that the differential effects for LXR α transactivation by full versus partial agonists might be related to their ability to recruit different levels of coactivators and corepressors, specifically NCoR. We found that reduced transcriptional efficacy observed for the partial agonists GW965 and GSK224 correlated to increased NCoR recruitment by the Gal4-DBD:LXR α -LBD fusion protein associated with a Gal-4 response element. We manipulated NCoR levels both by over-expression and knockdown and assessed the effect on LXR α transactivation on a DR4 element in reporter gene assays. These data suggested that relatively weak partial agonists such as GSK224 showed a strong preference to NCoR compared to a full agonist T09, which had little association with NCoR and preferred coactivators. Interestingly, the more modest partial agonist, GW965, could associate with both NCoR and coactivators, and its relative activity was highly dependent on the relative ratio or expression levels of NCoR.

We extended these studies to cellular gene expression assays in human liver hepatoma cells to determine if differences in transcriptional activity observed for these agonists in transactivation assays were recapitulated in vivo. For some of the genes we tested, including fatty acid synthase, we could observe gene expression profiles which strongly correlated with transcriptional efficacy observed in reporter gene assays. For FAS, we observed an ordered increase in gene expression, T09>GW965>GSK224 between full to weak partial agonists. However, another gene, SREBP1, showed robust activation in the presence of all three ligands, independent of full versus partial agonist. To explore whether these gene-specific expression profiles were related to NCoR recruitment, we tested NCoR occupancy at LXR response elements by chromatin immunoprecipitation. Indeed, NCoR recruitment to the FAS gene was highest for the weak partial agonist GSK224 and lowest for the full agonist T09. However, for SREBP1, NCoR recruitment was modest and showed no differences among the agonists. Thus, in vivo, the differential recruitment of NCoR induced by these agonists is gene-specific. It is likely that other transcriptional factors can influence activation and NCoR recruitment at genes such as SREBP1.

In chapter three, we explored the structural basis for repression of Rev-erb α by NCoR. Previously, the structural basis of SMRT-antagonism of PPAR α had been described. This data revealed that SMRT utilized a three-turn α -helical CoRNR box to mediate contact with PPAR α , which was consistent with a previously described extended form of

the CoRNR box (Nagy et al., 1999b; Xu et al., 2002). To better understand the molecular features of physiological repression by NRs, Rev-erb α , which functions as a constitutive repressor, was co-crystallized with a peptide containing the NCoR CoRNR box motif. In contrast to the antagonized PPAR α :SMRT structure, we observed that Rev-erb α :NCoR binding utilized a two-turn α -helical CoRNR box. This two-turn CoRNR motif was consistent with the description of a shorter, core CoRNR box motif originally proposed by our laboratory (Hu and Lazar, 1999). Thus, these receptors differed in their mode of corepressor binding. We next determined by scanning mutagenesis that these features observed in the Rev-erb:NCoR structure were indeed required for repression by Rev-erb α in cells. Altogether, this work provided novel insight into the structural basis of repression by NRs.

4.2 Future Directions

4.2a Selective Modulation of Nuclear Hormone Receptors

Nuclear hormone receptors constitute important therapeutic targets for endocrine, metabolic and circadian disorders. However, the varying roles of even the same receptor in multiple pathways, target tissues and on individual genes poses limitations in developing effective pharmacological agents that lack unwanted side effects. Original hypotheses from both a structural and functional standpoint were that NR LBDs functioned as ON and OFF switches, largely dependent on the positioning of helix 12. That is, in the liganded state, corepressors were dismissed and coactivators were fully

bound and genes were induced. In the OFF state, corepressors could be bound and gene expression was either at a basal or repressed state. However, our own studies with LXR as well as other reports suggest that NR transcriptional activity can be governed by a spectrum of relative coactivator and corepressor affinities. Surprisingly, our own partial agonist studies with LXR as well as studies with mice lacking function RID domains of NCoR suggest that full agonists for NRs such as LXR even induce a modest affinity for NCoR (Astapova et al., 2008). In these studies, a reduction in NCoR expression or function could further increase the activity of LXR even in the presence of a potent full agonist such as T09. Structurally, large-scale deuterium exchange studies of PPAR γ with a host of partial ligands provides evidence that subtle changes in LBD conformation independent of helix 12 can correlate to the relative transcriptional efficacy of partial agonists (Bruning et al., 2007). Thus, the ON/OFF switch hypothesis for NR activity misrepresents the true nature of NR activation whereby NRs may function more as a transcriptional “dimmer switch”, depending on ligand-induced conformational changes driving changes in cofactor affinities.

From our partial agonist studies, it is clear that we can effectively fine-tune transactivation potential of NRs pharmacologically on reporter genes. However, our in vivo data in cells suggests we have a very incomplete understanding of the nature of cofactor recruitment by NRs at specific genes. What cofactors are being recruited by liganded LXR at the SREBP1 promoter? What chromatin modifications are occurring at the SREBP1 promoter prior to and upon LXR activation? The genome-wide occupancy

studies by ChIP-CHIP and CHIP-SEQ have become available for a number of NRs including ER α and PPAR γ (Carroll et al., 2006; Lefterova et al., 2008b; Nielsen et al., 2008). These data sets provide unbiased, large-scale binding profiles for NRs, which has important implications in profiling pathways of regulation as well as the identification of specific target genes directly regulated by NRs. Application of these approaches to LXR α/β and its known cofactors including NCoR and SRC-1 for example, could delineate novel LXR target genes as well as the cofactor recruitment profile for LXR target genes. These types of analyses for example, could identify other transcription factors important for LXR activity, analogous to PPAR γ and C/EBPs, which might play critical roles in modulating LXR activity in a synergistic manner (Lefterova et al., 2008a). Next, secondary genome-wide profiling of NCoR and SRC-1 could identify the coregulator profiles associated with specific LXR target genes in human hepatoma cells in the presence of full versus partial agonists. Other approaches could include genome-wide profiling of chromatin modifications of LXR target genes under unliganded, full agonist and partial agonist conditions. Perhaps the chromatin context of certain LXR targets, such as SREBP1 differ from that of FAS, such that addition of the partial agonist GSK224, which otherwise has weak transcriptional efficacy, can result in gene activation profiles more similar to that of a full agonist. Combined with gene expression profiling, these methods can provide a systematic and complete interrogation of LXR binding, association with cofactors, and the chromatin environment at LXR target genes under different ligand conditions.

4.2b LXR as a Pharmacological Target

Due to its important roles in cholesterol metabolism and inflammation, LXR remains an important therapeutic target in cardiovascular disease. Pan-agonists of the LXR isoforms have been reported, but similar to our own studies, appear to induce lipogenic pathways in the liver (Fiévet and Staels, 2009) . Since LXR α is the predominant LXR isoform in the liver and is expressed at significantly higher levels in the liver than LXR β , one potential approach might be to selectively target LXR β .

To understand if targeting of LXR β might be sufficient to mediate physiological effects on peripheral cholesterol metabolism, mice lacking LXR α were crossed with ApoE-deficient mice, which can spontaneously develop atherosclerosis. These mice exhibited massive cholesterol accumulation in both peripheral target tissues and the liver, and the progression of atherosclerotic disease was significantly increased in the LXR α ^{-/-}/ApoE^{-/-} mice compared to ApoE^{-/-} controls. To explore the function of liganded LXR β in ameliorating this phenotype, these LXR α /ApoE-deficient mice were challenged with Western diet containing placebo or the potent LXR pan-agonist GW965 for approximately 30 days. Mice treated with LXR agonist showed dramatic decreases in peripheral cholesterol burden and demonstrated a potent induction of macrophage cholesterol efflux genes including ABCA1 (Bradley et al., 2007). In light of this study which suggests feasibility of LXR β targeting in atherosclerotic disease, there exists a large challenge in the design of an LXR β -selective agonist given the high degree of amino acid homology between the isoforms. However, recent reports suggests that

selectivity can be achieved with a novel class of non-steroidal ligands for LXR, N-acylthiadiazolines, so this strategy may be viable (Molteni et al., 2007).

4.2c Mechanisms of Ligand-Dependent Repression by Rev-erb α

The physiological significance of Rev-erb α repression has recently been demonstrated in NCoR mutant mice (Alenghat et al., 2008). These mice, which are unable to sufficiently repress Bmal1, have altered circadian period length and are also resistant to diet-induced obesity. Furthermore, the identification of the Rev-erb ligand heme (Raghuram et al., 2007; Yin et al., 2007), which facilitates recruitment of corepressor has also led to the report of a selective, synthetic ligand for Rev-erb receptors (Meng et al., 2008) This ligand was shown to directly affect the circadian clock phase through modulation of Rev-erb repressive activity. Given the role of Rev-erb receptors in regulating the positive limb of the circadian clock as well as regulation of physiological pathways including obesity, cholesterol homeostasis, gluconeogenesis and lipid metabolism, the pharmacological manipulation of Rev-erb activity in the treatment of biomedical disease is becoming increasingly plausible (Alenghat et al., 2008; Coste and Rodriguez, 2002; Duez et al., 2008; Raspe et al., 2002; Yin et al., 2007). Thus, an increased understanding of the structural basis for its repressive function is integral to pursuit of pharmacological targeting of this receptor.

Recently, several X-ray crystal structures of the highly related receptor Rev-erb β have been solved and reported (Pardee et al., 2009b; Woo et al., 2007). One structure reflects apo-Rev-erb β whereas the other structure reports the heme-bound Rev-erb β receptor. During this study it was discovered that indeed, Rev-erb α has a putative ligand, heme, and that binding of the ligand stabilized the receptor binding with its corepressor NCoR. Binding of the ligand was shown to be dependent on a critical histidine in the C-terminus of the ligand-binding domain, His⁶⁰². Efforts were made to co-crystallize a tertiary complex of Rev-erb α -LBD:NCoR-ID1:heme, but were ultimately unsuccessful. Interestingly, the report of Rev-erb β :heme crystal structure remarkably showed coordination of the analogous histidine, His⁵⁶⁸ as well as contacts with a cysteine, Cys³⁸⁴ contained within the hinge domain. The deletion mutant construct comprising Rev-erb α LBD used in this study did not contain this cysteine residue, Cys⁴¹⁷, and may explain an inability to co-crystallize the complex. Consistent with this, our preliminary experiments show that mutation of the analogous Cysteine, Cys⁴¹⁷ to Alanine significantly diminished the repression function of Rev-erb α , consistent with requirement of other residues to facilitate heme binding. This suggests that while His⁶⁰² is necessary for Rev-erb α :heme binding, it is likely not sufficient. Altogether, the receptor:heme:corepressor complex appears to be liganded by critical Histidine and cysteine residues necessary for heme binding which promote interaction between liganded Rev-erb α and the CoRNR box of NCoR.

Additional areas of investigation include the nature of heme liganding of the Rev-erb receptor in cells. Studies examining the intracellular levels of heme ligand indicate that heme levels oscillate with an 8-hour period (Rogers et al., 2008). The relative importance of both the eight-hour period, as well as the dynamic changes of heme concentration on Rev-erb α function are unknown. Furthermore, in vitro evidence suggests that heme may serve as a sensor for signaling gases such as NO. NO binding was reported to reduce Rev-erb repression, but the mechanism for this, i.e., changes in NCoR binding or NCoR function, are unknown (Pardee et al., 2009a).

4.2d Rev-erb β Function

Rev-erb β was initially cloned by the Moore laboratory based on the relative homology to other previously discovered nuclear hormone receptors (Dumas et al., 1994). This receptor shares ~95% homology with the Rev-erb α DNA Abinding domain and ~75% homology with the Rev-erb α ligand binding domain. There is little homology shared between the A/B (N-terminal domain) of these receptors, as well as reduced homology within the hinge region, which separates the DBD from the LBD. Thus, it is likely that some molecular targets of Rev-erb α may be conserved between the receptors. However, relative expression levels in various tissues as well as divergent regulation by post-translational modifications may likely mean these isoforms have unique functions depending upon the tissue or physiological context. Since its initial discovery, little has been published regarding the biological role of this receptor. In terms of circadian function, knockdown of this receptor was shown to affect Bmal1 amplitude in mouse

embryonic fibroblasts (Liu et al., 2008). It has been implicated to play a role in hepatic lipid metabolism via regulation of Apolipoprotein C-III, which was recently described to be dependent upon acetylation status of the DNA binding domain (Jiadong Wang, 2007; Wang et al., 2008). Most recently, the crystal structure of the ligand binding domain of this receptor has been reported by two independent groups, both in the apo state as well as complexed with its putative ligand, heme (Pardee et al., 2009a; Woo et al., 2007).

Preliminary evidence indicates that Rev-erb β contains repressive function on Rev-erb response elements including DR-2 and the mouse Bmal1 promoter. However, when compared to Rev-erb α , its repression function is markedly reduced, by 50% or more. The nature of this reduced repression function is unknown. Examination by ChIP of Rev-erb β binding to response elements in Bmal1 indicates a relative enrichment that is similar to that of Rev-erb α , suggesting the DNA binding activity of this isoform is intact. We also probed corepressor binding by immunoprecipitation methods and determined that binding of NCoR, SMRT and HDAC3 were robust. Binding of NCoR to Rev-erb β was also shown to be ligand-dependent, as heme depletion reduced NCoR association.

We also asked whether Rev-erb β might be differentially regulated by post-translational modifications which might be affecting its repression function. Sumoylation, which occurs on lysine residues, has been shown to critically regulate the activity of a number of transcription factors and coregulators including nuclear hormone receptors as well as

the critical clock component Bmal1 (Cardone et al., 2005; Chen et al., 2004; Lee et al., 2008). We performed an in silico scan of putative sumoylation residues and discovered that Rev-erb β contains a perfect sumoylation consensus motif within its N-terminal A/B domain. The repression function of this and other potential sumoylation sites was examined using lysine to arginine substitution at these putative residues. Interestingly, the A/B domain sumoylation mutant, K68R, had dramatically increased repression function, suggesting that Rev-erb β repressive activity can be affected by amino acid substitutions within the A/B domain. When we co-expressed factors necessary for sumoylation, we also observed an apparent molecular weight shift of Rev-erb β protein, consistent with our hypothesis that Rev-erb β might be sumoylation target. Recent reports that PPAR α sumoylation status alters the ability of the receptor to bind NCoR (Pourcet et al., 2009), further illustrates the potential for post-translational modifications such as sumoylation to be playing important roles in regulation of NR activity

Interestingly, recent reports suggest there may be novel cofactors recruited by Rev-erb β (Jiadong et al., 2007; Wang et al., 2008). It will be of interest to confirm binding of these putative cofactors and test their ability to alter Rev-erb β transcriptional activity. Furthermore, given the decreased repression function of Rev-erb β observed in reporter assays, a recent report has suggested that Rev-erb β may even activate transcription of SREBP2 (Ramakrishnan et al., 2009). Thus, future experiments can be performed to identify the molecular mechanism for reduced Rev-erb β function, as well as the potential role of post-translational modifications, such as sumoylation, on Rev-erb β function. In

the longer term, it also will be important to address via ChIP-CHIP or ChIP-SEQ methods the putative target genes for Rev-erb β , and the pathways which are co-regulated by the Rev-erb receptors or specific to Rev-erb β .

REFERENCES

- Ackerman, M.N., Friedman, R.A., Doyle, W.J., Bluestone, C.D., and Fireman, P. (1984). Antigen-induced eustachian tube obstruction: an intranasal provocative challenge test. *J Allergy Clin Immunol* 73, 604-609.
- Albers, M., Blume, B., Schlueter, T., Wright, M.B., Kober, I., Kremoser, C., Deuschle, U., and Koegl, M. (2006). A Novel Principle for Partial Agonism of Liver X Receptor Ligands. *Journal of Biological Chemistry* 281, 4920-4930.
- Alenghat, T., Meyers, K., Mullican, S.E., Leitner, K., Adeniji-Adele, A., Avila, J., Bucan, M., Ahima, R.S., Kaestner, K.H., and Lazar, M.A. (2008). Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. *Nature* 456, 997-1000.
- Aranda, A., and Pascual, A. (2001). Nuclear Hormone Receptors and Gene Expression. *Physiol Rev* 81, 1269-1304.
- Astapova, I., Lee, L.J., Morales, C., Tauber, S., Bilban, M., and Hollenberg, A.N. (2008). The nuclear corepressor, NCoR, regulates thyroid hormone action in vivo. *Proceedings of the National Academy of Sciences* 105, 19544-19549.
- Bain, D.L., Heneghan, A.F., Connaghan-Jones, K.D., and Miura, M.T. (2007). Nuclear Receptor Structure: Implications for Function. *Annual Review of Physiology* 69, 201-220.
- Baniahmad, A., Kohne, A.C., and Renkawitz, R. (1992). A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. *EMBO J* 11, 1015-1023.
- Beato, M. (1989). Gene regulation by steroid hormones. *Cell* 56, 335-344.
- Beato, M., Herrlich, P., and Schütz, G. (1995). Steroid hormone receptors: Many Actors in search of a plot. *Cell* 83, 851-857.
- Berger, S.L. (2007). The complex language of chromatin regulation during transcription. *Nature* 447, 407-412.
- Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., *et al.* (2006). A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell* 125, 315-326.
- Bradley, M.N., Hong, C., Chen, M., Joseph, S.B., Wilpitz, D.C., Wang, X., Lusis, A.J., Collins, A., Hseuh, W.A., Collins, J.L., *et al.* (2007). Ligand activation of LXR² reverses atherosclerosis and cellular cholesterol overload in mice lacking LXR¹ and apoE. *The Journal of Clinical Investigation* 117, 2337-2346.

- Bruning, J.B., Chalmers, M.J., Prasad, S., Busby, S.A., Kamenecka, T.M., He, Y., Nettles, K.W., and Griffin, P.R. (2007). Partial Agonists Activate PPAR[gamma] Using a Helix 12 Independent Mechanism. *Structure* 15, 1258-1271.
- Cardone, L., Hirayama, J., Giordano, F., Tamaru, T., Palvimo, J.J., and Sassone-Corsi, P. (2005). Circadian Clock Control by SUMOylation of BMAL1. *Science* 309, 1390-1394.
- Carroll, J.S., Liu, X.S., Brodsky, A.S., Li, W., Meyer, C.A., Szary, A.J., Eeckhoute, J., Shao, W., Hestermann, E.V., Geistlinger, T.R., *et al.* (2005). Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 122, 33-43.
- Carroll, J.S., Meyer, C.A., Song, J., Li, W., Geistlinger, T.R., Eeckhoute, J., Brodsky, A.S., Keeton, E.K., Fertuck, K.C., Hall, G.F., *et al.* (2006). Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* 38, 1289-1297.
- Casanova, J., Helmer, E., Selmi-Ruby, S., Qi, J.S., Au-Fliegner, M., Desai-Yajnik, V., Koudinova, N., Yarm, F., Raaka, B.M., and Samuels, H.H. (1994). Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor. *Molecular & Cellular Biology* 14, 5756-5765.
- Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y., Burris, T.P., and Rastinejad, F. (2008). Structure of the intact PPAR-[ggr]-RXR-[agr] nuclear receptor complex on DNA. *Nature*, 350-356.
- Chatterjee, V.K.K., Nagaya, T., Madison, L.D., Datta, S., Rentoumis, A., and Jameson, J.L. (1991). Thyroid hormone resistance syndrome. Inhibition of normal receptor function by mutant thyroid hormone receptors. *J Clin Invest* 87, 1977-1984.
- Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999a). Regulation of transcription by a protein methyltransferase. *Science* 284, 2174-2177.
- Chen, H., Lin, R.J., Xie, W., Wilpitz, D., and Evans, R.M. (1999b). Regulation of hormone-induced histone hyperacetylation and gene activation by acetylation of an acetylase. *Cell* 98, 675-686.
- Chen, J.D., and Evans, R.M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 377, 454-457.
- Chen, W.-Y., Lee, W.-C., Hsu, N.-C., Huang, F., and Chung, B.-c. (2004). SUMO Modification of Repression Domains Modulates Function of Nuclear Receptor 5A1 (Steroidogenic Factor-1). *J Biol Chem* 279, 38730-38735.
- Chiang, J.Y., Kimmel, R., and Stroup, D. (2001). Regulation of cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription by the liver orphan receptor (LXRalpha). *Gene* 262, 257-265.

Chomez, P., Neveu, I., Mansen, A., Kiesler, E., Larsson, L., Vennstrom, B., and Arenas, E. (2000). Increased cell death and delayed development in the cerebellum of mice lacking the rev-erb α orphan receptor. *Development* 127, 1489-1498.

Coste, H., and Rodriguez, J.C. (2002). Orphan Nuclear Hormone Receptor Rev-erb α Regulates the Human Apolipoprotein CIII Promoter. *Journal of Biological Chemistry* 277, 27120-27129.

Coste, H., and Rodriguez, J.C. (2002). Orphan nuclear hormone receptor Rev-erb α regulates the human apolipoprotein CIII promoter. *J Biol Chem* 277, 27120-27129.

Costet, P., Luo, Y., Wang, N., and Tall, A.R. (2000). Sterol-dependent Transactivation of the ABC1 Promoter by the Liver X Receptor/Retinoid X Receptor. *Journal of Biological Chemistry* 275, 28240-28245.

Danielian, P.S., White, R., Lees, J.A., and Parker, M.G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J* 11, 1025-1033.

Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J., and Yamamoto, K.R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev* 12, 3343-3356.

Desvergne, B., Michalik, L., and Wahli, W. (2006). Transcriptional Regulation of Metabolism. *Physiol Rev* 86, 465-514.

Downes, M., Carozzi, A.J., and Muscat, G.E.O. (1995). Constitutive expression of the orphan receptor, Rev-ErbA α , inhibits muscle differentiation and abrogates the expression of the *myoD* gene family. *Mol Endocrinol* 9, 1666-1678.

Duez, H., and Staels, B. (2008). Rev-erb[α] gives a time cue to metabolism. *FEBS Letters* 582, 19-25.

Duez, H., van der Veen, J.N., Duhem, C., Pourcet, B., Touvier, T., Fontaine, C., Derudas, B., Baugé, E., Havinga, R., Bloks, V.W., *et al.* (2008). Regulation of Bile Acid Synthesis by the Nuclear Receptor Rev-erb[α]. *Gastroenterology* 135, 689-698.e685.

Dumas, B., Harding, H., Choi, H.-S., Lehmann, K., Chung, M., Lazar, M.A., and Moore, D.D. (1994). A new orphan member of the nuclear hormone receptor superfamily closely related to Rev-Erb. *Mol Endocrinol* 8, 996-1005.

Egea, P.F., Klaholz, B.P., and Moras, D. (2000). Ligand-protein interactions in nuclear receptors of hormones. *FEBS Letters* 476, 62-67.

Feng, W., Ribeiro, R.C.J., Wagner, R.L., Nguyen, H., Apriletti, J.W., Fletterick, R.J., Baxter, J.D., Kushner, P.J., and West, B.L. (1998). Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* 280, 1747-1749.

- Fiévet, C., and Staels, B. (2009). Liver X receptor modulators: Effects on lipid metabolism and potential use in the treatment of atherosclerosis. *Biochemical Pharmacology* 77, 1316-1327.
- Ghisletti, S., Huang, W., Ogawa, S., Pascual, G., Lin, M.E., Willson, T.M., Rosenfeld, M.G., and Glass, C.K. (2007). Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. *Mol Cell* 25, 57-70.
- Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 14, 121-141.
- Green, S.P., Walter, P., Kumar, B., Krust, A., Bornert, J.-M., Argos, P.K., and Chambon, P. (1986). Human estrogen receptor cDNA: sequence, expression, and homology to v-erbA. *Nature* 320, 134-139.
- Grefhorst, A., Elzinga, B.M., Voshol, P.J., Plosch, T., Kok, T., Bloks, V.W., van der Sluijs, F.H., Havekes, L.M., Romijn, J.A., Verkade, H.J., *et al.* (2002). Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem* 277, 34182-34190.
- Gronemeyer, H., Gustafsson, J.-A., and Laudet, V. (2004). Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov* 3, 950-964.
- Guenther, M.G., Barak, O., and Lazar, M.A. (2001). The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. *Mol Cell Biol* 21, 6091-6101.
- Guenther, M.G., Lane, W.S., Fischle, W., Verdin, E., Lazar, M.A., and Shiekhhattar, R. (2000). A core SMRT corepressor complex containing HDAC3 and a WD40 repeat protein linked to deafness. *Genes Dev* 14, 1048-1057.
- Guenther, M.G., Yu, J., Kao, G.D., Yen, T.J., and Lazar, M.A. (2002). Assembly of the SMRT-histone deacetylase 3 repression complex requires the TCP-1 ring complex. *Genes Dev* 16, 3130-3135.
- Harding, H.P., and Lazar, M.A. (1995). The monomer-binding orphan receptor Rev-Erb represses transcription as a dimer on a novel direct repeat. *Mol Cell Biol* 15, 4791-4802.
- Hartman, H.B., Yu, J., Alenghat, T., Ishizuka, T., and Lazar, M.A. (2005). The histone-binding code of nuclear receptor co-repressors matches the substrate specificity of histone deacetylase 3. *EMBO Rep* 6, 445-451.
- He, B., Gampe Jr, R.T., Kole, A.J., Hnat, A.T., Stanley, T.B., An, G., Stewart, E.L., Kalman, R.I., Mingos, J.T., and Wilson, E.M. (2004). Structural Basis for Androgen Receptor Interdomain and Coactivator Interactions Suggests a Transition in Nuclear Receptor Activation Function Dominance. *Molecular Cell* 16, 425-438.

- Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387, 733-736.
- Hollenberg, S.M., Weinberger, C., Ong, E.S., G.Cerelli, Oro, A., Lebo, R., Thompson, E.B., Rosenfeld, M.G., and Evans, R.M. (1985). Primary structure and expression of a functional glucocorticoid receptor cDNA. *Nature* 318, 635-641.
- Horlein, A.J., Naar, A.M., Heinzl, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C.K., *et al.* (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* 377, 397-404.
- Hu, E., Kim, J.B., Sarraf, P., and Spiegelman, B.M. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR γ . *Science* 274, 2100-2103.
- Hu, X., and Lazar, M.A. (1999). The CoRNR motif controls the recruitment of corepressors to nuclear hormone receptors. *Nature* 402, 93-96.
- Hu, X., and Lazar, M.A. (2000). Transcriptional repression by nuclear hormone receptors. *Trends Endocrinol Metab* 11, 6-10.
- Hu, X., Li, S., Wu, J., Xia, C., and Lala, D.S. (2003). Liver x receptors interact with corepressors to regulate gene expression. *Mol Endocrinol* 17, 1019-1026.
- Hu, X., Li, Y., and Lazar, M.A. (2001). Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. *Mol Cell Biol* 21, 1747-1758.
- Ishikawa, T., Umesono, K., Mangelsdorf, D.J., Aburatani, H., Stanger, B.Z., Shibasaki, Y., Imawari, M., Evans, R.M., and Takaku, F. (1990). A functional retinoic acid receptor encoded by the gene on human chromosome 12. *Mol Endocrinol* 4, 837-844.
- Ishizuka, T., and Lazar, M.A. (2003). The N-CoR/HDAC3 deacetylase complex is required for repression by thyroid hormone receptor. *Mol Cell Biol* 23, 5122-5131.
- Ishizuka, T., and Lazar, M.A. (2005). The nuclear receptor corepressor deacetylase activating domain is essential for repression by thyroid hormone receptor. *Mol Endocrinol* 19, 1443-1451.
- Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R., and Mangelsdorf, D.J. (1996). An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383, 728-731.
- Jaye, M.C., Krawiec, J.A., Campobasso, N., Smallwood, A., Qiu, C., Lu, Q., Kerrigan, J.J., De Los Frailes Alvaro, M., Laffitte, B., Liu, W.-S., *et al.* (2005). Discovery of Substituted Maleimides as Liver X Receptor Agonists and Determination of a Ligand-Bound Crystal Structure. *Journal of Medicinal Chemistry* 48, 5419-5422.

- Jepsen, K., Gleiberman, A.S., Shi, C., Simon, D.I., and Rosenfeld, M.G. (2008). Cooperative regulation in development by SMRT and FOXP1. *Genes Dev* 22, 740-745.
- Jepsen, K., Harmanson, O., Onami, T.M., Gleiberman, A.S., Lunyak, V., McEvelly, R.J., Kurokawa, R., Kumar, V., Liu, F., Seto, E., *et al.* (2000). Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell* 102, 753-763.
- Jiadong, W., Yang, L., Min, Z., Zhongle, L., Cong, W., Hanying, Y., Yu-Yang, L., Xin, Z., and Hong, L. (2007). A zinc finger HIT domain-containing protein, ZNHIT-1, interacts with orphan nuclear hormone receptor Rev-erb β ; and removes Rev-erb β -induced inhibition of apoCIII transcription. *FEBS Journal* 274, 5370-5381.
- Jiadong Wang, Y.L.M.Z.Z.L.C.W.H.Y.Y.-Y.L.X.Z.H.L. (2007). A zinc finger HIT domain-containing protein, ZNHIT-1, interacts with orphan nuclear hormone receptor Rev-erb β ; and removes Rev-erb β -induced inhibition of apoCIII transcription. *FEBS Journal* 274, 5370-5381.
- Joseph, S.B., Castrillo, A., Laffitte, B.A., Mangelsdorf, D.J., and Tontonoz, P. (2003). Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med* 9, 213-219.
- Joseph, S.B., McKilligin, E., Pei, L., Watson, M.A., Collins, A.R., Laffitte, B.A., Chen, M., Noh, G., Goodman, J., Hagger, G.N., *et al.* (2002). Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A* 99, 7604-7609.
- Kemper, J.K., Xiao, Z., Ponugoti, B., Miao, J., Fang, S., Kanamaluru, D., Tsang, S., Wu, S.-Y., Chiang, C.-M., and Veenstra, T.D. (2009). FXR Acetylation Is Normally Dynamically Regulated by p300 and SIRT1 but Constitutively Elevated in Metabolic Disease States. *Cell Metabolism* 10, 392-404.
- Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., and Bass, J. (2007). High-Fat Diet Disrupts Behavioral and Molecular Circadian Rhythms in Mice. *J Biol Chem* 282, 414-421.
- Kuwabara, M.D., and Sigman, D.S. (1987). *Biochemistry* 26, 7234-7238.
- Laffitte, B.A., Repa, J.J., Joseph, S.B., Wilpitz, D.C., Kast, H.R., Mangelsdorf, D.J., and Tontonoz, P. (2001). LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc Natl Acad Sci U S A* 98, 507-512.
- Lazar, M.A., Jones, K.E., and Chin, W.W. (1989). Isolation of a cDNA encoding human Rev-ErbA α : Transcription from the non-coding strand of a thyroid hormone receptor gene results in a related protein which does not bind thyroid hormone. *Nuc Acids Res* 17, 101-109.

- Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B.t., Sasso, G.L., Moschetta, A., and Schibler, U. (2009). REV-ERB β Participates in Circadian SREBP Signaling and Bile Acid Homeostasis. *PLoS Biol* 7, e1000181.
- Lee, J., Lee, Y., Lee, M.J., Park, E., Kang, S.H., Chung, C.H., Lee, K.H., and Kim, K. (2008). Dual Modification of BMAL1 by SUMO2/3 and Ubiquitin Promotes Circadian Activation of the CLOCK/BMAL1 Complex. *Mol Cell Biol* 28, 6056-6065.
- Lee, K.K., and Workman, J.L. (2007). Histone acetyltransferase complexes: one size doesn't fit all. *Nat Rev Mol Cell Biol* 8, 284-295.
- Lefstin, J.A., Thomas, J.R., and Yamamoto, K.R. (1994). Influence of a steroid receptor DNA-binding domain on transcriptional regulatory functions. *Genes Dev* 8, 2842-2856.
- Lefterova, M.I., Zhang, Y., Steger, D.J., Schupp, M., Schug, J., Cristancho, A., Feng, D., Zhuo, D., Stoeckert, C.J., Jr., Liu, X.S., *et al.* (2008a). PPAR γ and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes Dev* 22, 2941-2952.
- Lefterova, M.I., Zhang, Y., Steger, D.J., Schupp, M., Schug, J., Cristancho, A., Feng, D., Zhuo, D., Stoeckert, C.J., Liu, X.S., *et al.* (2008b). PPAR γ and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes & Development* 22, 2941-2952.
- Lehmann, J.r.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.-L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A., *et al.* (1997). Activation of the Nuclear Receptor LXR by Oxysterols Defines a New Hormone Response Pathway. *Journal of Biological Chemistry* 272, 3137-3140.
- Levin, N., Bischoff, E.D., Daige, C.L., Thomas, D., Vu, C.T., Heyman, R.A., Tangirala, R.K., and Schulman, I.G. (2005). Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler Thromb Vasc Biol* 25, 135-142.
- Liu, A.C., Tran, H.G., Zhang, E.E., Priest, A.A., Welsh, D.K., and Kay, S.A. (2008). Redundant Function of REV-ERB α and β and Non-Essential Role for Bmal1 Cycling in Transcriptional Regulation of Intracellular Circadian Rhythms. *PLoS Genet* 4, e1000023.
- Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., and Sigler, P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352, 497-505.
- Luo, Y., and Tall, A.R. (2000). Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *The Journal of Clinical Investigation* 105, 513-520.
- Mader, S., Chen, J.Y., Chen, Z., White, J., Chambon, P., and Gronemeyer, H. (1993). The patterns of binding of RAR, RXR, and TR homo- and heterodimers to direct repeats

are dictated by the binding specificities of the DNA binding domains. *EMBO J* *12*, 5029-5041.

Mangelsdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. *Cell* *83*, 841-850.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., *et al.* (1995). The nuclear receptor superfamily: the second decade. *Cell* *83*, 835-839.

McEwan, I.J. (2004). Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. *Endocr Relat Cancer* *11*, 281-293.

McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Kronen, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., *et al.* (1998). Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. *Genes Dev* *12*, 3357-3368.

Meijsing, S.H., Pufall, M.A., So, A.Y., Bates, D.L., Chen, L., and Yamamoto, K.R. (2009). DNA Binding Site Sequence Directs Glucocorticoid Receptor Structure and Activity. *Science* *324*, 407-410.

Meng, Q.J., McMaster, A., Beesley, S., Lu, W.Q., Gibbs, J., Parks, D., Collins, J., Farrow, S., Donn, R., Ray, D., *et al.* (2008). Ligand modulation of REV-ERB{alpha} function resets the peripheral circadian clock in a phasic manner. *J Cell Sci* *121*, 3629-3635.

Miao, B., Zondlo, S., Gibbs, S., Cromley, D., Hosagrahara, V.P., Kirchgessner, T.G., Billheimer, J., and Mukherjee, R. (2004). Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator. *J Lipid Res* *45*, 1410-1417.

Miyajima, N., Horiuchi, R., Shibuya, Y., Fukushige, S., Matsubara, K., Toyoshima, K., and Yamamoto, T. (1989). Two erbA homologs encoding proteins with different T3 binding capacities are transcribed from opposite DNA strands of the same genetic locus. *Cell* *57*, 31-39.

Molteni, V., Li, X., Nabakka, J., Liang, F., Wityak, J., Koder, A., Vargas, L., Romeo, R., Mitro, N., Mak, P.A., *et al.* (2007). N-Acylthiadiazolines, a New Class of Liver X Receptor Agonists with Selectivity for LXR β . *Journal of Medicinal Chemistry* *50*, 4255-4259.

Moras, D., and Gronemeyer, H. (1998). The nuclear receptor ligand-binding domain: structure and function. *Curr Opin Cell Biol* *10*, 384-391.

- Nagy, L., Kao, H.-Y., Love, J.D., Li, C., Banayo, E., Gooch, J.T., Krishna, V., Chatterjee, K., Evans, R.M., and Schwabe, J.W.R. (1999a). Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes & Development* *13*, 3209-3216.
- Nagy, L., Kao, H.Y., Love, J.D., Li, C., Banayo, E., Gooch, J.T., Krishna, V., Chatterjee, K., Evans, R.M., and Schwabe, J.W. (1999b). Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes Dev* *13*, 3209-3216.
- Nagy, L., and Schwabe, J.W. (2004). Mechanism of the nuclear receptor molecular switch. *Trends Biochem Sci* *29*, 317-324.
- Nettles, K.W., and Greene, G.L. (2005). LIGAND CONTROL OF COREGULATOR RECRUITMENT TO NUCLEAR RECEPTORS. *Annual Review of Physiology* *67*, 309-333.
- Nielsen, R., Pedersen, T.Å.s., Hagenbeek, D., Moulos, P., SiersbÃk, R., Megens, E., Denissov, S., BÃrgesen, M., Francoijs, K.-J., Mandrup, S., *et al.* (2008). Genome-wide profiling of PPARÎ³:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. *Genes & Development* *22*, 2953-2967.
- Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K., and Milburn, M.V. (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. *Nature* *395*, 137-143.
- Ogawa, S., Lozach, J., Benner, C., Pascual, G., Tangirala, R.K., Westin, S., Hoffmann, A., Subramaniam, S., David, M., Rosenfeld, M.G., *et al.* (2005). Molecular Determinants of Crosstalk between Nuclear Receptors and Toll-like Receptors. *Cell* *122*, 707-721.
- Oonate, S.A., Tsai, S.Y., Tsai, M.-J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* *270*, 1354-1357.
- Osborne, C.K., Zaho, H., and Fuqua, S.A. (2000). Selective estrogen receptor modulators: structure, function, and clinical use. *J Clin Oncol* *18*, 3172-3186.
- Pardee, K.I., Xu, X., Reinking, J., Schuetz, A., Dong, A., Liu, S., Zhang, R., Tiefenbach, J., Lajoie, G., Plotnikov, A.N., *et al.* (2009a). The Structural Basis of Gas-Responsive Transcription by the Human Nuclear Hormone Receptor REV-ERBβ. *PLoS Biology* *7*, e43.
- Pardee, K.I., Xu, X., Reinking, J., Schuetz, A., Dong, A., Liu, S., Zhang, R., Tiefenbach, J., Lajoie, G., Plotnikov, A.N., *et al.* (2009b). The Structural Basis of Gas-Responsive Transcription by the Human Nuclear Hormone Receptor REV-ERBÎ². *PLoS Biol* *7*, e1000043.

- Pascual, G., Fong, A.L., Ogawa, S., Gamliel, A., Li, A.C., Perissi, V., Rose, D.W., Willson, T.M., Rosenfeld, M.G., and Glass, C.K. (2005). A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature*, 1.
- Pascual, G., and Glass, C.K. (2006). Nuclear receptors versus inflammation: mechanisms of transrepression. *Trends Endocrinol Metab* 17, 321-327.
- Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E., and Mangelsdorf, D.J. (1998). Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 93, 693-704.
- Perissi, V., Staszewski, L.M., McInerney, E.M., Kurokawa, R., Kronen, A., Rose, D.W., Lambert, M.H., Milburn, M.V., Glass, C.K., and Rosenfeld, M.G. (1999). Molecular determinants of nuclear receptor-corepressor interaction. *Genes Dev* 13, 3198-3208.
- Pourcet, B., Pineda-Torra, I.s., Derudas, B., Staels, B., and Glineur, C. (2009). SUMOylation of the human peroxisome proliferator-activated receptor alpha inhibits its trans-activity through the recruitment of the nuclear corepressor NCoR. *Journal of Biological Chemistry*, -.
- Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110, 251-260.
- Privalsky, M.L. (2004). The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu Rev Physiol* 66, 315-360.
- Quinet, E.M., Savio, D.A., Halpern, A.R., Chen, L., Miller, C.P., and Nambi, P. (2004). Gene-selective modulation by a synthetic oxysterol ligand of the liver X receptor. *J Lipid Res* 45, 1929-1942.
- Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P., and Rastinejad, F. (2007). Identification of heme as the ligand for the orphan nuclear receptors REV-ERB[alpha] and REV-ERB[beta]. *Nat Struct Mol Biol* 14, 1207-1213.
- Ramakrishnan, S.N., Lau, P., Crowther, L.M., Cleasby, M.E., Millard, S., Leong, G.M., Cooney, G.J., and Muscat, G.E.O. (2009). Rev-erb beta regulates the Srebp-1c promoter and mRNA expression in skeletal muscle cells. *Biochemical and Biophysical Research Communications* 388, 654-659.
- Rangwala, S.M., Rhoades, B., Shapiro, J.S., Rich, A.S., Kim, J.K., Shulman, G.I., Kaestner, K., and Lazar, M.A. (2003). Genetic modulation of PPARgamma phosphorylation regulates insulin sensitivity. *Developmental Cell* 5, 6570663.

- Raspe, E., Duez, H., Mansen, A., Fontaine, C., Fievet, C., Fruchart, J.C., Vennstrom, B., and Staels, B. (2002). Identification of Rev-erb α as a physiological repressor of apoC-III gene transcription. *J Lipid Res* 43, 2172-2179.
- Rastinejad, F., Perlmann, T., Evans, R.M., and Sigler, P.B. (1995). Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature* 375, 203-211.
- Rastinejad, F., Wagner, T., Zhao, Q., and Khorasanizadeh, S. (2000). Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. *EMBO J* 19, 1045-1054.
- Renaud, J.-P., Harris, J.M., Downes, M., Burke, L.J., and Muscat, G.E.O. (2000). Structure-Function Analysis of the Rev-erbA and RVR Ligand-Binding Domains Reveals a Large Hydrophobic Surface That Mediates Corepressor Binding and a Ligand Cavity Occupied by Side Chains. *Mol Endocrinol* 14, 700-717.
- Repa, J.J., Turley, S.D., Lobaccaro, J.A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R.A., Dietschy, J.M., and Mangelsdorf, D.J. (2000). Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 289, 1524-1529.
- Rogers, P.M., Ying, L., and Burris, T.P. (2008). Relationship between circadian oscillations of Rev-erb α expression and intracellular levels of its ligand, heme. *Biochemical and Biophysical Research Communications* 368, 955-958.
- Ruthenburg, A.J., Allis, C.D., and Wysocka, J. (2007). Methylation of Lysine 4 on Histone H3: Intricacy of Writing and Reading a Single Epigenetic Mark. *Molecular Cell* 25, 15-30.
- Saaticioglu, F., Bartunek, P., Deng, T., Zenke, M., and Karin, M. (1993). A conserved C-terminal sequence that is deleted in v-ErbA is essential for the biological activities of c-ErbA (the thyroid hormone receptor). *Mol Cell Biol* 13, 3675-3685.
- Schmidt, R.J., Ficorilli, J.V., Zhang, Y., Bramlett, K.S., Beyer, T.P., Borchert, K., Dowless, M.S., Houck, K.A., Burris, T.P., Eacho, P.I., *et al.* (2006). A 15-ketosterol is a liver X receptor ligand that suppresses sterol-responsive element binding protein-2 activity. *J Lipid Res* 47, 1037-1044.
- Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D.J., *et al.* (2000). Role of LXRs in control of lipogenesis. *Genes Dev* 14, 2831-2838.
- Shang, Y., and Brown, M. (2002). Molecular determinants for the tissue specificity of SERMs. *Science* 295, 2465-2468.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95, 927-937.

shown, d.n.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. *Nature* 403, 41-45.

Tangirala, R.K., Bischoff, E.D., Joseph, S.B., Wagner, B.L., Walczak, R., Laffitte, B.A., Daige, C.L., Thomas, D., Heyman, R.A., Mangelsdorf, D.J., *et al.* (2002). Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci U S A* 99, 11896-11901.

Teboul, M., Guillaumond, F., Grechez-Cassiau, A., and Delaunay, F. (2008). Minireview: The Nuclear Hormone Receptor Family Round the Clock. *Mol Endocrinol* 22, 2573-2582.

Tong, G.-X., Jeyakumar, M., Tanen, M.R., and Bagchi, M.K. (1996). Transcriptional silencing by unliganded thyroid hormone receptor β requires a soluble corepressor that interacts with the ligand-binding domain of the receptor. *Mol Cell Biol* 16, 1909-1920.

Tontonoz, P., and Mangelsdorf, D.J. (2003). Liver X receptor signaling pathways in cardiovascular disease. *Mol Endocrinol* 17, 985-993.

Traves, P.G., Hortelano, S., Zeini, M., Chao, T.-H., Lam, T., Neuteboom, S.T., Theodorakis, E.A., Palladino, M.A., Castrillo, A., and Bosca, L. (2007). Selective Activation of Liver X Receptors by Acanthoic Acid-Related Diterpenes. *Molecular Pharmacology* 71, 1545-1553.

Trojer, P., and Reinberg, D. (2006). Histone Lysine Demethylases and Their Impact on Epigenetics. *Cell* 125, 213-217.

Tsai, M., and O'Malley, B.W. (1994). Molecular Mechanisms of Action of Steroid/Thyroid Receptor Superfamily Members. *Annu Rev Biochem* 63, 451-486.

Umesono, K., and Evans, R.M. (1989). Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* 57, 1139-1146.

Wade, P.A., and Wolffe, A.P. (1999). Transcriptional regulation: SWItching circuitry. *Current Biology* 9, R221-R224.

Wang, J., and Lazar, M.A. (2008). Bifunctional role of Rev-erb alpha in adipocyte differentiation. *Molecular and Cellular Biology* 28, 2213-2220.

Wang, J., Liu, N., Liu, Z., Li, Y., Song, C., Yuan, H., Li, Y.-y., Zhao, X., and Lu, H. (2008). The orphan nuclear receptor Rev-erb[beta] recruits Tip60 and HDAC1 to regulate

apolipoprotein CIII promoter. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1783, 224-236.

Wang, J., Yin, L., and Lazar, M.A. (2006). The orphan nuclear receptor Rev-erb alpha regulates circadian expression of plasminogen activator inhibitor type 1. *J Biol Chem* 281, 33842-33848.

Wei, L.N., Hu, X., Chandra, D., Seto, E., and Farooqui, M. (2000). Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. *J Biol Chem* 275, 40782-40787.

Wen, Y.D., Perissi, V., Staszewski, L.M., Yang, W.M., Kroner, A., Glass, C.K., Rosenfeld, M.G., and Seto, E. (2000). The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc Natl Acad Sci USA* 97, 7202-7207.

Westin, S., Kurokawa, R., Nolte, R.T., Wisely, G.B., McInerney, E.M., Rose, D.W., Milburn, M.V., Rosenfeld, M.G., and Glass, C.K. (1998). Interactions controlling the assembly of nuclear receptor heterodimers and co-activators. *Nature* 395, 199-202.

Williams, S., Bledsoe, R.K., Collins, J.L., Boggs, S., Lambert, M.H., Miller, A.B., Moore, J., McKee, D.D., Moore, L., Nichols, J., *et al.* (2003). X-ray crystal structure of the liver X receptor beta ligand binding domain: regulation by a histidine-tryptophan switch. *J Biol Chem* 278, 27138-27143.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Mangelsdorf, D.J. (1995). LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev* 9, 1033-1045.

Wilson, T.E., Paulsen, R.E., Padgett, K.A., and Milbrandt, J. (1992). Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. *Science* 256, 107-110.

Woo, E.-J., Jeong, D.G., Lim, M.-Y., Jun Kim, S., Kim, K.-J., Yoon, S.-M., Park, B.-C., and Eon Ryu, S. (2007). Structural Insight into the Constitutive Repression Function of the Nuclear Receptor Rev-erb[beta]. *Journal of Molecular Biology* 373, 735-744.

Wu, N., Yin, L., Hanniman, E.A., Joshi, S., and Lazar, M.A. (2009). Negative feedback maintenance of heme homeostasis by its receptor, Rev-erb1. *Genes & Development* 23, 2201-2209.

Wu, Y., and Koenig, R.J. (2000). Gene regulation by thyroid hormone. *Trends Endocrinol Metab* 11, 207-211.

Wurtz, J.M., Bourguet, W., Renaud, J.P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996). A canonical structure for the ligand binding domain of nuclear receptors. *Nature Struct Biol* 3, 87-94.

- Xu, H.E., Stanley, T.B., Montana, V.G., Lambert, M.H., Shearer, B.G., Cobb, J.E., McKee, D.D., Galardi, C.M., Plunket, K.D., Nolte, R.T., *et al.* (2002). Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPAR α . *Nature* *415*, 813-817.
- Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Straume, M., Mangelsdorf, D.J., and Evans, R.M. (2006). Nuclear receptor expression links the circadian clock to metabolism. *Cell* *126*, 801-810.
- Yang, X., Lamia, K.A., and Evans, R.M. (2007). Nuclear Receptors, Metabolism, and the Circadian Clock. *Cold Spring Harbor Symposia on Quantitative Biology* *72*, 387-394.
- Yin, L., and Lazar, M.A. (2005). The orphan nuclear receptor Rev-erb α recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian *Bmal1* gene. *Mol Endocrinol* *19*, 1452-1459.
- Yin, L., Wang, J., Klein, P.S., and Lazar, M.A. (2006). Nuclear receptor Rev-erb α is a critical lithium-sensitive component of the circadian clock. *Science* *311*, 1002-1005.
- Yin, L., Wu, N., Curtin, J.C., Qatanani, M., Szwegold, N.R., Reid, R.A., Waitt, G.M., Parks, D.J., Pearce, K.H., Wisely, G.B., *et al.* (2007). Rev-erb α , a Heme Sensor That Coordinates Metabolic and Circadian Pathways. *Science* *318*, 1786-1789.
- Yoh, S.M., Chatterjee, V.K.K., and Privalsky, M.L. (1997). Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. *Mol Endocrinol* *11*, 470-480.
- Yoh, S.M., and Privalsky, M.L. (2000). Resistance to thyroid hormone (RTH) syndrome reveals novel determinants regulating interaction of T3 receptor with corepressor. *Mol Cell Endocrinol* *159*, 109-124.
- Yu, J., Li, Y., Ishizuka, T., Guenther, M.G., and Lazar, M.A. (2003). A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. *EMBO J* *22*, 3403-3410.
- Zamir, I., Dawson, J., Lavinsky, R.M., Glass, C.K., Rosenfeld, M.G., and Lazar, M.A. (1997a). Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex. *Proc Natl Acad Sci USA* *94*, 14400-14495.
- Zamir, I., Zhang, J., and Lazar, M.A. (1997b). Stoichiometric and steric principles governing repression by nuclear hormone receptors. *Genes Dev* *11*, 835-846.
- Zhang, L., Rubins, N.E., Ahima, R.S., Greenbaum, L.E., and Kaestner, K.H. (2005). *Foxa2* integrates the transcriptional response of the hepatocyte to fasting. *Cell Metabolism* *2*, 141-148.

Zhao, Q., Khorasanizadeh, S., Miyoshi, Y., Lazar, M.A., and Rastinejad, F. (1998). Structural elements of an orphan nuclear receptor-DNA complex. *Molecular Cell* 1, 849-861.