Chapter 6
Nuclear Receptors: Small Molecule Sensors that Coordinate Growth, Metabolism and Reproduction

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Abstract One of the largest groups of metazoan transcription factors (TFs), the Nuclear Receptor superfamily, regulates genes required for virtually all aspects of development, reproduction and metabolism. Together, these master regulators can be thought of as a fundamental operating system for metazoan life. Their most distinguishing feature is a structurally conserved domain that acts as a switch, powered by the presence of small diffusible ligands. This ligand-responsive regulation has allowed the Nuclear Receptors to help their hosts adapt to a wide variety of physiological niches and roles, making them one of the most evolutionarily successful TF families. Originally discovered as receptors for steroid hormones, the Nuclear Receptor field has grown to encompass much more than traditional endocrinology. For example, recent work has highlighted the role of Nuclear Receptors as major regulators of metabolism and biological clocks. By monitoring endogenous metabolites and absorbed xenobiotics, these receptors also coordinate rapid, system-wide responses to changing metabolic and environmental states. While many new Nuclear Receptor ligands have been discovered in the past couple of decades, approximately half of the 48 human receptors are still orphans, with a significantly higher percentage of orphans in other organisms. The discovery of new ligands has led to the elucidation of new regulatory mechanisms, target genes, pathways and functions. This review will highlight both the common as well as newly emerging traits and functions that characterize this particularly unique and important TF family.

6.1 Introduction

The ability of Nuclear Receptor (NR) ligands to move relatively freely within the body, tissues and cells sets the NRs apart from other signaling systems. G

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T.R. Hughes (ed.), *A Handbook of Transcription Factors*, Subcellular Biochemistry 52,
DOI 10.1007/978-90-481-9069-0_6,

protein-coupled receptors, for example, must transduce their ligand signals from the cell surface to the nucleus through an extended chain of intermediary signaling proteins. Accordingly, the coupling of ligand-binding and DNA-binding capabilities allows NRs to directly sense the concentration of their ligands within the cell and at target gene promoters [1, 2].

NRs have been shown to act as key regulators in a diverse range of developmental and homeostatic pathways. These include embryogenesis, growth, vascular tone, detoxification, circadian rhythm, glucose and lipid homeostasis, reproduction and behavior. Consistent with their role in most, if not all, fundamental biological processes, mutations in NR genes also play a role in most human disease states including obesity, inflammation, autoimmune disorders, cardiovascular disease and cancer [3, 4]. Importantly, many of these disease states can be prevented or treated by the use of natural or synthetic NR ligands. However, only a relatively small number of all NRs have a known natural ligand. As a consequence, in an effort to identify novel therapeutics, significant attempts have been made towards the identification of both endogenous and synthetic ligands for the orphan members of the NR superfamily [5, 6].

6.2 Nuclear Receptor Domain Architecture

Early work on NRs revealed a common domain architecture, traditionally referred to by the letters A-F, from the N- to C-terminal ends respectively (Fig. 6.1). However, only the C and E domains are broadly conserved. The C domain encompasses the highly conserved DNA binding domain (DBD), and the E domain the less well conserved ligand-binding domain (LBD). These are linked by a flexible hinge region (D domain), which varies in length and sequence. Like the D domain, the A/B domains are also poorly conserved and relatively unstructured [7]. This N-terminal region is also often referred to as the Activation Function-1 (AF-1) domain, due to its general role in transcriptional activation. Some receptors also contain an extended carboxyl-terminal domain, referred to as the F-domain, which appears to have a general role in transcriptional repression [8, 9]. While there is interaction and interdependence between these domains, these regions are largely semi-autonomous and modular [10].

Fig. 6.1 Nuclear receptor domain structure. Beginning at the N-terminus, nuclear receptors include the N-terminal domain (A/B), DNA binding domain (C), Hinge region (D), ligand binding domain (E) and C-terminal domain (F)

6.3 NR Phylogeny and Classes

NRs have been shown to exist in most metazoan clades, including sponges, echinoderms, tunicates, arthropods and vertebrates, and are therefore believed to be present throughout the Metazoa. As such the NR superfamily represents one of the largest families of transcriptional regulators in metazoans [11–13]. Comparison of the DBD and LBD sequences has led to the classification of NRs into six subfamilies (Table 6.1) [14]. These are denoted by the prefix "NR" followed by a three-digit code. The first digit refers to the subfamily number, the second digit to the group letter and the final digit to the specific gene number [15]. As an example, the receptor Rev-erbα was given the code NR1D1, which corresponds to subfamily I, group D, and being the first gene identified in this group. This nomenclature also encompasses insect receptors. For example the *Drosophila* homolog to Rev-erbα, E75, is identified by the code NR1D3.

6.4 NR Evolution

A major question regarding the evolution of NRs is how they managed to gain the ability to bind ligands that are functionally relevant to the genes they control. For example, in most of the characterized examples, ligands are either metabolic precursors, products or targets of the gene products regulated by the receptor. Clues to this conundrum have been provided by the presence of NR genes and target genes in more primitive organisms, and the NR sequences themselves.

NRs are not found in plants, fungi or protozoa. However, if the DBD and LBD are considered separately, the yeast zinc-finger homologs containing LIM (eg: PXL1) and GATA (eg: GZF3, GATs 1-4 and DAL80) domains, and the yeast membrane protein Pex11p, have partial sequence and structural alignment with these respective NR domains. Based on these similarities, it has been proposed that NRs may have arisen from the fusion of these or related proteins in pre-metazoan eukaryotes as early as 635 million years ago [11, 24, 25]. Another protein family with interesting functional parallels to the NRs is the fungal binuclear zinc cluster TFs. Like the NRs, this family of fungal proteins can operate as monomers or dimers and is modulated by small molecules, including nutrients, metabolites and xenobiotics. The domain structure of these proteins is also remarkably similar to the NRs, beginning with an N-terminal zinc cluster DBD followed by a linker and LBD [26, 27].

Species at the base of the metazoan clade have provided further insight into the NR ancestral state [11, 28, 29]. The earliest metazoan species, the sponges, only have receptors from subfamily II (ie: HNF4/RXR), while moderately early metazoans, such as *Hydra sp.* and *Anemonia sp.,* contain a larger number of subfamily II receptors (Coup-tf, TLL, TR2/4) and a putative member of subfamily VI (GCNF ortholog) [28, 30, 31]. However, all six NR subfamilies are found within most other levels of metazoan phylogeny, suggesting that they underwent their first

Table 6.1 Summary of human nuclear receptors with their known endogenous ligands and *Drosophila* homologs

Subfamily 1: Thyroid hormone receptor-like

Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
A	Thyroid hormone receptor	TRα	NR1A1	Thyroid hormone	
		TRβ	NR1A2	Thyroid hormone	
B	Retinoic acid receptor	RARα	NR1B1	Retinoic acid	
		RARβ	NR1B2	Retinoic acid	
		RARγ	NR1B3	Retinoic acid	
C	Peroxisome proliferator-activated receptor	PPARα	NR1C1	Fatty acids, leukotriene B4	
		PPARβ/δ	NR1C2	Fatty acids	
		PPARγ	NR1C3	Fatty acids	
D	Rev-erbα and β	Rev-erbα	NR1D1	Heme, NO, CO	E75 (80/25, Heme, NO, CO), E78 (69/23)
		Rev-erbβ	NR1D2	Heme, NO, CO	
F	RAR-related orphan receptor	RORα	NR1F1	Cholesterol, cholesterol sulphate	DHR3 (76/35, Cholesterol)
		RORβ	NR1F2	All trans retinoic acid	
		RORγ	NR1F3	Retinoic acid	
H	Liver X receptor	LXRα	NR1H3	Oxysterols	EcR (64/37, 20-hydroxyecdysone)
		LXRβ	NR1H2	Oxysterols	
	Farnesoid X receptor	FXR	NR1H4	Bile acids, lanosterol	EcR (72/28, 20-hydroxyecdysone)
I	Vitamin D receptor	VDR	NR1I1	Vitamin D_3, bile acids	DHR96 (55/20, Cholesterol)
	Pregnane X receptor	PXR	NR1I2	Xenobiotics	
	Constitutive androstane receptor	CAR	NR1I3	Androstane, xenobiotics	

Table 6.1 (continued)

Subfamily 2: Retinoid X receptor-like

Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
A	Hepatocyte nuclear factor-4	HNF4α	NR2A1	Fatty acids	DHNF4 (89/61)
		HNF4γ	NR2A2	Fatty acids	
B	Retinoid X receptor	RXRα	NR2B1	9-cis-Retinoic acid; Heme?	Ultraspiracle (84/43, Phosphatidyl-ethanolamine)
		RXRβ	NR2B2	9-cis-Retinoic acid	
		RXRγ	NR2B3	9-cis-Retinoic acid	
C	Testicular receptor	TR2	NR2C1	Orphan	DHR78 (67/23)
		TR4	NR2C3	Orphan	
E	Human homologue of the *Drosophila* tailless gene	TLX	NR2E1	Orphan	Tailless (80/34), Dissatisfaction (74/35)
	Photoreceptor cell-specific nuclear receptor	PNR	NR2E3	Orphan	DHR51 (70/47, Heme, NO, CO) DNR83 (60/20)
F	Chicken ovalbumin upstream promoter-transcription factor	COUP-TFI	NR2F1	Orphan	Seven up (89/92)
		COUP-TFII	NR2F2	Orphan	
	V-erbA-related gene IV-erbA-related	EAR-2	NR2F6	Orphan	

Subfamily 3: Estrogen receptor-like

Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
A	Estrogen receptor	ERα	NR3A1	Estradiol-17β	
		ERβ	NR3A2	Estradiol-17β	
B	Estrogen-related receptor	ERRα	NR3B1	Orphan	DERR (88/34)
		ERRβ	NR3B2	Orphan	
		ERRγ	NR3B3	Orphan	
C	Glucocorticoid receptor	GR	NR3C1	Cortisol	
	Mineralocorticoid receptor	MR	NR3C2	Aldosterone	

Table 6.1 (continued)

	Progesterone receptor	PR	NR3C3	Progesterone	
	Androgen receptor	AR	NR3C4	Testosterone	
Subfamily 4: Nerve growth factor IB-like					
Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
A	Nerve growth FACTOR IB	NGFIB	NR4A1	Orphan	
	Nuclear receptor related 1	NURR1	NR4A2	Orphan	DHR38 (93/59)
	Neuron-derived orphan receptor 1	NOR1	NR4A3	Orphan	
Subfamily 5: Steroidogenic factor-like					
Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
A	Steroidogenic factor 1	SF1	NR5A1	Phospholipid	Ftz-f1 (88/28), DHR39 (60/26)
	Liver receptor homolog-1	LRH-1	NR5A2	Phospholipid	Ftz-f1 (89/35), DHR39 (62/25)
Subfamily 6: Germ cell nuclear factor-like					
Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
A	Germ cell nuclear factor	GCNF	NR6A1	Orphan	DHR4 (61/21)
Subfamily 0: Miscellaneous					
Group	Human receptor	Isoform	Symbol	Endogenous ligand	Fly ortholog (%ID DBD/LBD, ligand)
B	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	DAX1	NR0B1	Orphan	
	Small heterodimer partner	SHP	NR0B2	Orphan	
C	Nuclear receptors with two DNA binding domains	2DBD-NR		Orphan	

Data for Table 6.1 compiled from [1, 16–23].

round of diversification before the metazoans experienced significant phylogenetic radiation. The second wave of vertebrate NR diversification occurred much later and followed the divergence of invertebrate and vertebrate lineages. This event generated the paralogous groups and much of the diversity observed within human receptors. It is this multiplicity that has led to isoform-specific tissue expression, function and ligand binding profiles for many of the vertebrate receptors [11, 16, 32].

The current consensus is that the primordial NR was not ligand regulated. Instead this feature appears to have developed independently multiple times during evolutionary history. Although receptors may have first evolved as apo-proteins, it has been suggested that the first "ligands" were permanent co-factors. Recalling that the common ancestral NR may have belonged to subfamily II [29], this makes a lot of sense. This subfamily contains HNF4 and USP, two receptors that appear to bind non-exchangeable structural co-factors rather than conventional ligands [23, 33–35]. If the LBD of the common ancestral receptor relied on a co-factor, this constraint may have contributed to the structural conservation we see in LBDs across the receptor group. Furthermore, such an ancestral receptor provides a plausible model for the development of reversible ligand binding. The exchange of such a co-factor with structurally similar compounds from the cellular environment may have been the origin of ligand binding [11, 36, 37]. As one possible example, the NRD1 orthologue in flies, E75, requires heme as a structural component [19], whereas the vertebrate counterparts, the Rev-erbs, can exchange heme readily with no apparent effects on stability [22, 38, 39].

6.5 Co-evolution of Receptors and Ligands

A genome-wide comparison of NR/ligand pairs has also led to the understanding that in many cases there is no correlation between receptor subfamilies and the biosynthetic origin of their ligands. The most dramatic example of this can be found in subfamily I. Although the TRs, RARs, PPARs and VDRs are all closely related by sequence, their ligands are all synthesized in highly divergent metabolic pathways and differ highly in structure (Table 6.1). The same lack of correlation is also apparent with the RXR and RAR receptors. Although these bind structurally and biosynthetically similar ligands, 9-*cis* retinoic acid and all-*trans* retinoic acid respectively, they are some of the most distantly related human receptors [11, 40].

A few key observations can be made based on the distribution of receptors and ligands. Most importantly, NRs and their ligands did not, in general, co-evolve. Second, this lack of correlation between receptor and ligand suggests that the original coupling of receptors and ligands likely resulted from beneficial fortuitous interactions [11, 40, 41]. However, there is evidence that once the receptor/ligand pair was functionally coupled, ligand binding specificity and affinity may have co-evolved through mutations to either the receptor or the enzymes of ligand biosynthesis [42]. The vertebrate steroid receptors provide an interesting example. The ancestral steroid receptor is the Estrogen Receptor (ER), which can be found in both protostome and deuterostome species. From this one primordial

steroid receptor, vertebrate-specific diversification gave rise to the six variants in present day vertebrates. Genome sequence analyses suggests that these transitions were accompanied by the evolution of steroidogenic and steroid-specific catabolic enzymes, producing new potential ligands and genes for the new paralogs to bind and regulate [43].

Interestingly, *Drosophila* appears to have lost the original steroid-binding receptors. However, it has developed a parallel steroidal signaling system centered around ecdysteroids and the subfamily I receptor, Ecdysone Receptor (EcR) [40, 42]. It appears that the EcR and other sterol binding members of subfamily I likely evolved from a common ancestor, referred to as proto-FXR/LXR/EcR, which acquired the ability to bind steroids independent of the subfamily III steroid receptors [32, 40]. The insect EcR from subfamily I serves a functionally parallel role to the vertebrate sex steroid receptors of subfamily III in vertebrates. So remarkably, both vertebrates and invertebrates have evolved independent steroid-based developmental NRs [1, 44].

6.6 DBD Structure and Function

The DBD serves as a gene locator for the receptor by docking to specific hexanucleotide sequences or response elements (REs) in the promoter/enhancer regions of gene targets. Sequence conservation is highest in the DBD, which is due presumably to a need to conserve binding site specificity as well as structural stability within such a small domain [9, 37]. Making up the core of the DBD are two zinc finger motifs, each containing four cysteine residues that together coordinate a single zinc atom. These cysteine–zinc interactions stabilize the domain in place of a hydrophobic core. The N-terminal helix of the DBD interacts with the major groove of the DNA, and thus it is this sequence, called the P-box, which defines the DNA binding specificity of the receptor. The second helix lies perpendicular to helix 1, and contributes to domain stability and dimerization with the partner DBD.

NRs have been categorized into four classes based on their mode of DNA binding (Fig. 6.2) [45]. The first, class I, defines the mechanism of action for the steroid receptors. For most class I NRs, ligand binding occurs in the cytoplasm, which triggers the shedding of chaperones and translocation to the nucleus. Once there, they bind to inverted hexanucleotide repeats as homodimers in a head-to-head configuration. Class II NRs form heterodimers with RXR and bind to hexanucleotide direct repeats in a head-to-tail configuration. These NRs bind to DNA independent of ligand status. As apo-receptors, they silence gene expression, and in the presence of a ligand, transcription is activated [36, 45]. Many of the NRs in classes III and IV are orphans, and not surprisingly, remain less understood and more heterogeneous. Like class I receptors, class III receptors homodimerize but only bind promoters with hexameric direct repeats. Class IV receptors can bind as either monomers or dimers, but are unique in that they bind only single hexameric sites. As the orphans become better understood their regulatory

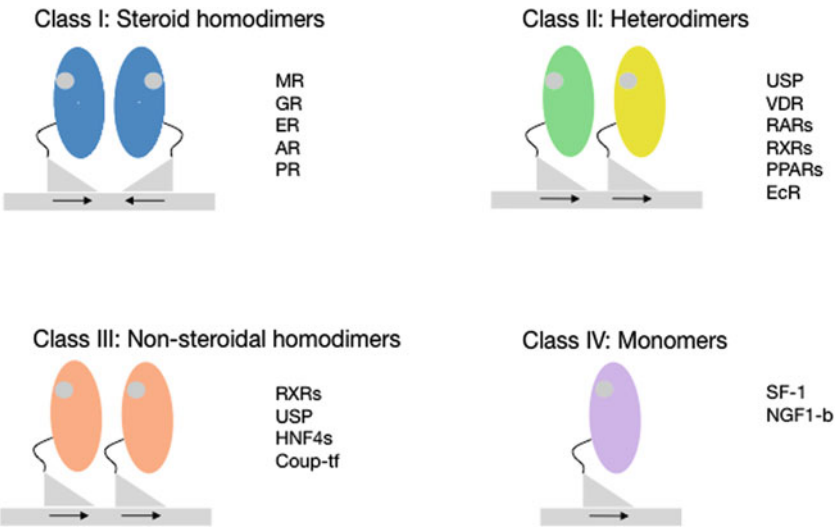

Fig. 6.2 Four Nuclear Receptor classes based on DNA response elements. Nuclear receptors can be grouped into four classes according to their ligand binding, DNA binding, and dimerization properties: steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors. Steroid receptors bind to DNA at inverted response element (RE) repeats as homodimers. RXR heterodimers bind to DNA at direct RE repeats. Homodimeric orphan receptors bind to DNA at direct RE repeats. Monomeric orphan receptors bind to single REs as individual monomers. Shown are representative receptors for each group with known ligands. Adapted from [45]

features will likely become even more enmeshed with the class I and II receptors [9, 16, 36, 37].

Given the high sequence similarity between the hexameric REs recognized by NR DBDs, a key factor in determining their target gene specificity is the orientation and spacing of REs within promoters [9, 16, 36, 46]. In the absence of dimerization, NR monomers derive further DNA specificity through interactions between a DNA-binding motif C-terminal of the DBD (called the C-terminal extension) and DNA sequence immediately 5′ of the hexameric RE. For many NR monomers, this interaction contributes significant specificity and stability to DNA binding [47–50]. Even so, further specificity cues are required in vivo to discriminate between the tens or hundreds of thousands of potential binding sites and those that are functional. Recent genome-wide binding studies suggest that the average NR is bound to a subset of approximately 5,000–10,000 binding sites within a particular cell type, approximately 10% of which contribute to changes in gene expression levels (reviewed in [51]). These studies also suggest that additional cell-specific target gene selection is provided by differences in chromatin accessibility and cofactors that help tether and stabilize NRs on active sites [52–56].

Although the classical view of NR DNA binding holds that the DBD is responsible only for site-specific recognition and binding to DNA, it should be noted that

recent evidence from structural studies has indicated that binding of the DBD to specific RE sequences may be more than just a mechanism for localizing the receptor to the correct DNA sequence [57]. In fact, it has been hypothesized that the exact hexanucleotide sequence of the RE affects not only the overall affinity of the receptor for its RE site, but also influences the three dimensional (3-D) configuration of the receptor, thereby regulating NR activity through the binding of certain ancillary factors [57, 58]. Recent studies have also shown that interactions between different NR domains can influence DNA contacts and binding site specificity [59].

6.7 LBD Structure and Functional Classifications

The LBD, as the name indicates, is responsible for binding of the receptor's cognate ligand(s) and can be thought of as a molecular switch that mediates the transcription output. Although the sequence conservation of this domain between receptors can be as low at 15%, the 3-D structure is nonetheless universally conserved. The secondary structure, in most solved structures, is composed of 12 helices and three short β-strands. Described as an α-helical sandwich, the LBD comprises three antiparallel layers of helices that form the sides and central layer of the fold (Fig. 6.3) [10]. The central and generally hydrophobic core of this globular domain is absent in the lower half of the domain, and it is this non-polar cavity that forms the ligand-binding pocket. The sides of this pocket are composed of the outer layers of the α-helical sandwich, and the front and back are formed by helix 12 and two to three β-strands, respectively.

As alluded to earlier, the LBD also serves as a primary mediator for the self-assembly of receptors into homo or heterodimers. This dimerization, mediated primarily by helices 9 and 10, contributes to the specificity of DNA binding by correctly spacing and orienting the DBD subunits [9, 60]. Interestingly, while dimerization interfaces between respective LBDs and DBDs are well established, the importance of interdomain contacts was only recently shown with the solution of the first intact DBD/LBD dimer structure. In this PPARγ/RXRα structure, the DBD and LBD of the opposite heterodimer partners also form dimerization interfaces that contribute to the stability of the complex [59].

Another critical function of the LBD is to serve as a platform for the binding and assembly of transcriptional co-activator or co-repressor complexes. These proteins are recruited or shed from the LBD surface depending on the ligand-binding state of the domain. Early crystal structures showed that much of the structural basis for these transitions lies with the positioning of helix 12, which moves in the presence of ligand to close off the ligand binding pocket, and to redesign the cofactor binding grooves. In the absence of ligand, co-repressors containing LXXI/HIXXXI/L motifs (also referred to as a co-repressor nuclear-receptor [CoRNR] box) can bind, whereas in the presence of an agonist ligand, co-activators containing LXXLL motifs can bind [9, 10]. An example of such interactions is clearly demonstrated by the apo-, agonist- and antagonist-bound forms

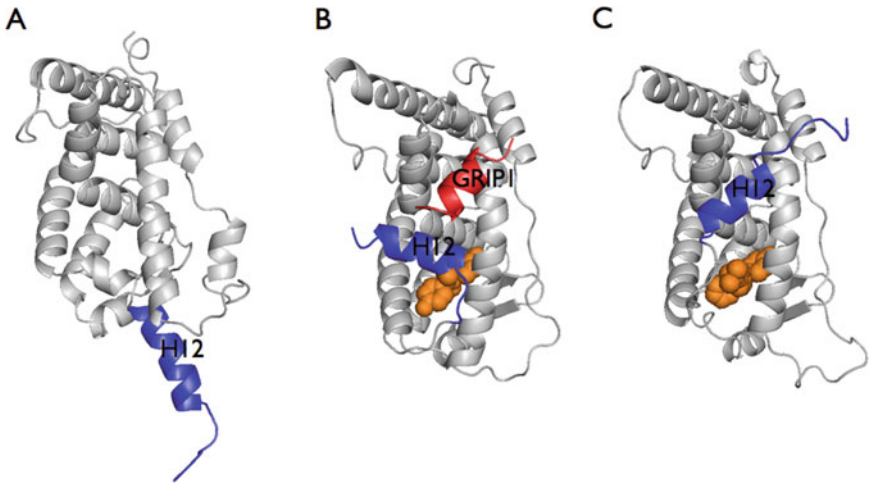

Fig. 6.3 Structural basis for ligand-response. The structures shown demonstrate the canonical apo, agonist-bound and antagonist-bound conformations of NR LBDs [63]. (**a**) The unliganded form of RXRα shows H12 extending away from the body of the LBD and H11 partially occupying the ligand binding pocket (RXRα; PDB: 1LDB). (**b**) Agonist bound ERα, in association with a co-activator GRIP1 peptide, is in a transcriptionally active conformation (ERα/diethylstilbestrol; PDB 3ERD; [64]). (**c**) Antagonist bound ERα in a transcriptionally inactive conformation. The molecular extension of 4-hydroxytamoxifen protrudes from the ligand-binding pocket to displace AF-2/helix 12, which instead occupies the hydrophobic groove and blocks co-activator binding (ERα/4-hydroxytamoxifen; PDB 3ERT; [64]). Helix 12 is in *blue*, the GRIP1 peptide is in *red*, agonist/antagonist ligands are in *orange* and the main body of the LBD is in *grey*

of the ERα and RAR LBDs (Fig. 6.3). Co-repressors tend to recruit chromatin condensing complexes, whereas co-activators recruit chromatin opening and RNA polymerase II holoenzyme recruiting complexes [61]) (Fig. 6.4). Although the majority of well-characterized cofactors are those that interact with the LBD, there are also numerous NR–specific interactions made by the variable A/B domains. Comprehensive reviews on NR cofactors have recently been published [61, 62].

As orphan receptors have become adopted, the conventionality of this LBD response has been challenged. The human receptors CARβ and RORβ, for instance, appear to be constitutively active in the absence of a ligand. In what could be described as the inverse of the NR model, their respective endogenous ligands androstane and all-trans retinoic acid, repress the high basal transcription levels of the apo receptors [65, 66]. There are also likely to be many NRs that require a ligand in order to fully repress their target genes, as appears to be the case for the NR1D receptors (E75 and Rev-erbs). These NRs lack the canonical helix 12, and appropriately, appear to function as dedicated repressors. As more ligands are discovered, further variations on the theme defined by the steroid receptors are likely to be discovered.

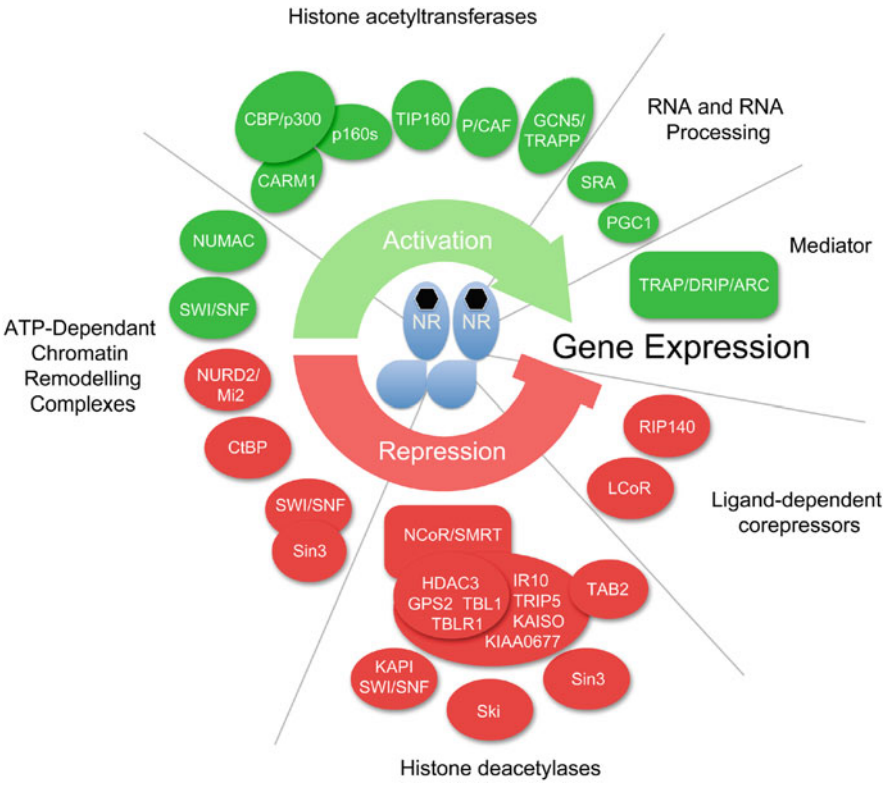

Fig. 6.4 Coactivator and corepressor complexes. Coactivator complexes (*green*) include factors that contain ATP-dependent chromatin remodeling histone arginine methyltransferase and histone acetyltransferase activities. They may also contain factors involved in RNA processing as well as components of the Mediator complex. Conversely, co-repressors (*red*) recruit histone deacetylases and other chromatin-condensing enzymes and cofactors. Adapted from [67]

6.8 NR Ligands

Ligands for NRs are small and hydrophobic, giving them the general ability to move relatively freely between tissues and cells. The binding of hydrophobic ligands also contributes to the stability of the cognate receptor, completing the LBD hydrophobic core and setting up further intra- and inter-molecular interactions [10, 68]. The ligand responsive nature of NRs has meant that, long before the advent of modern pharmacology, NRs have been probed by the chemical diversity surrounding them. These interactions include fortuitous ecological compounds, and now man-made pollutants, as well as compounds actively synthesized by plants and animals for chemical defence. An intriguing example of this is the fly EcR and the evolution of ecdysteroids outside of insect taxa. Ecdysteroids are the steroids responsible for the timing of development in insects [1, 44]. However, ecdysteroids have also been identified in many sessile species, such as soft coral and many plants,

at concentrations 2–5 fold greater than what is found in insects. While these organisms do not contain an EcR, they synthesize precise chemical mimics of the insect ecdysteroid 20-hydroxyecdysone (20E), as well as many other biologically active variations of the insect hormone. This inappropriate EcR activation disrupts the insects developmental program, resulting in lethality [69–72].

As evidenced by the use of plants as the source of our first pharmaceuticals, the ability of natural products to mimic the structure of receptor ligands is not limited to interactions with the insect world. Some examples of interactions with mammalian NRs are the isoflavones (phytoestrogens) from legumes, which interact with the ER, and have been shown to reduce the risk of certain cancers and heart disease [73]. The plant sterol guggulsterone is another interesting example, in which interaction with FXR has been shown to reduce serum cholesterol in mammals [74]. Interestingly ecdysteroids also have a physiological effect on mammals, with positive effects on muscle strength, lipid metabolism and immunity being some of the most cited [75]. Such interactions hold both risk to human health, as in the case of endocrine disruptors, and potential benefit in the form of new drugs. Accordingly, NR-based drug discovery and toxicology screens are actively probing the natural environment for interacting compounds [76–78]. These interactions are discussed in greater detail below (section 6.12).

Many NR drugs do not simply agonize or antagonize the receptor, but have pharmacological selectivity that comes from the disruption of specific receptor/co-regulator interactions that are either responsible for only a subset of receptor functions or are cell-type specific [16]. One of the best-studied examples of these selective nuclear receptor modulators (SNuRMs) is the anti-cancer drug tamoxifen. While tamoxifen serves as an antagonist to combat ER positive cancer in breast tissue, it conversely serves as an ER agonist in the bone and uterus, where ER activity is still needed. The tissue-specific nature of this response is, at least in part, the result of differential co-factor distribution, with the co-activator SRC1 at higher levels in the uterus and bone [15, 16, 79, 80]. Similar drugs are being developed for other NRs, and hold promise for overcoming the side effects of current treatments. Such new selective modulators provide a model for not only the next generation of NR drugs, but may also inspire similar strategies for other therapeutic targets [5, 81].

With the discoveries of recently de-orphaned NR ligands has come the realization of new ligand types and interaction mechanisms. Some of the first endocrine NRs to be characterized, such as ER, TR and VDR, were found to bind their ligands with high affinity, but also to readily release or exchange their ligands. The more recently deorphanized metabolic NRs tend to bind physiologically abundant ligands, with a lower affinity. Several other NRs, such as HNF4α/γ and the fly NRs E75 and USP, bind molecules that appear to serve as permanent co-factors or prosthetic groups [19, 23, 33–35]. This diversity of ligand types and interactions is continuing to grow. One of the most surprising and unusual is the E75/Rev-erb ligand heme and its retention in the LBD pocket via coordinate bonds between the heme iron and amino acid side-chains. Even more unusual is the ability of E75/Rev-erb-heme to bind the diatomic gases Nitric oxide and Carbon monoxide, which displace one of

the coordinate bonds [19, 22, 38, 39]. The unconventional and unexpected nature of these new ligands may explain, in part, the recalcitrance of the remaining orphans to reveal their ligand identities. There are almost certainly new surprises waiting in the wings.

6.9 The Orphan Receptors

Several solved LBD structures lack a ligand-binding pocket, which has led to the suggestion that many orphan NRs may be authentic orphan receptors with no ligand counterpart. For example, Nurr1, and its fly homolog DHR38 [82, 83], and Rev-erb β [84], when purified from bacterial expression systems, contained no ligand or pocket. Accordingly, it was suggested that these LBDs function simply as transcriptionally active platforms for constitutive cofactor binding [16, 68, 85]. This interpretation was strengthened by observations that these and other NR LBDs can recruit cofactors in the absence of ligand. One consequence of these findings has been a decrease in drug development programs directed against NRs by the major pharmaceutical companies.

Recent studies, however, have challenged this commonly held point of view. For example, the Rev-erb LBDs have since been shown to be capable of binding heme, with significant LBD structural changes made to accommodate this relatively large molecule [22]. This type of flexibility, in terms of LBD pocket size and shape, has also been found with a number of other NRs [22, 86–89]. Thus, not only may orphan LBDs exist in apo and bound forms, but some may also be capable of binding multiple and diverse ligand types, as observed with PXR [90] and EcR [86]. These ligands could serve as agonists or antagonists that elicit both quantitative and qualitative differences in activities. An exciting consideration is that some of these ligands may exist only in certain tissues, with unique outcomes on cofactor recruitment, target gene selection and ensuing levels of expression.

6.10 Other Modes of Nuclear Receptor Activity Modulation

Beyond the ligand-binding pocket, there are many established ligand-independent modes of control that influence the transcriptional activity of NRs. As with other TFs, post-translational modifications are a significant contributor to NR responses and responsiveness. Phosphorylation, sumoylation, ubiquitynation and acetylation can all influence receptor stability, localization and cofactor interactions [36, 68, 91–93]. The widespread nature of post-translational signaling and the growing recognition of ligand-independent modes of control have led to calls to broaden research efforts beyond the LBD towards the consideration of a “multivalent allosteric switch” that reacts to a wide range of inputs [16]. The details of these alternative modes of regulation, however, are likely to be receptor-specific in terms of the degree and mechanism of action, whereas LBD–ligand interactions will tend to have more universal and pervasive consequences.

6.11 Nuclear Receptor Functions

NRs have generally been classified functionally into one of two groups, endocrine or metabolic/xenobiotic. However, it is becoming increasingly clear that NRs have a large number of functions that bridge these broad roles, as well as many others. In fact, these initial classifications are relatively uninformative and misleading. Nevertheless, for historical and clarification purposes, these groups are described below.

The endocrine receptors, which include the Androgen Receptor (AR), Mineralocorticoid Receptor (MR), Glucocorticoid Receptor (GR), Progesterone Receptor (PR), ERα and ERβ are largely recognized for their roles in developmental and reproductive biology [16]. Orthologues for these NRs in *Drosophila*, *C. elegans* and *Ciona*, are largely absent. In fact, aside from the orphan dERRs, the steroid subfamily III is completely absent from these genomes [40]. When this absence was first noted, it was assumed that steroid receptors must be a product of vertebrate evolution [12, 32]. However, as mentioned earlier, ER orthologues are found in early metazoans. Thus the fly and worm clade, termed the Ecdysozoans, appears to have undergone loss of all but one subfamily III steroid receptor gene [13].

The metabolic and xenobiotic sensors, comprised of the PPARs, FXRs, LXRs, RORs, Rev-erbs, and HNF4s, allow organisms to respond to metabolic imbalances and changes in their environment. Ligands for these receptors are often nutritionally important compounds or intermediates and products of key biochemical pathways [3]. Together, these receptors have been shown to form a network that ensures energy and metabolic homeostasis [22, 38, 39, 94, 95] (Fig. 6.5). In their surveillance of metabolism, many of these NRs regulate the genes involved in the production, destruction or trafficking of their own ligands. For example, to aid in metabolite clearance, metabolic NRs upregulate catalytic enzymes, such as P450s, to transform excess compounds into less active/more soluble intermediates. These same receptors also promote pathways and transporters involved in the ultimate elimination of these compounds [96–98].

The xenobiotic receptors (PXR, CAR, ERRs) form a parallel system that monitors the chemical diversity surrounding the organism for chemical threats in the environment. In the same way the metabolic receptors respond to an oversupply of endogenous metabolites, these receptors respond to toxic threats by upregulating catabolic enzymes and transporters [3–5, 99]. Together, these two receptor systems, metabolic and xenobiotic, form a sensing and response network throughout the body, and are particularly important in the gut. As one of the largest interfaces with the outside chemical world, these NRs help the enteric tract cue genetic responses to our changing nutritional status as well as pathogenic and toxic challenges [94, 100].

It has been 20 years since the first metabolic NRs were identified. The RXR/RARs and the PPARs were found to bind retinoic acid and fatty acid metabolites, respectively [101–104]. In the time since, an entire subgroup of receptors has been identified as being regulated by endogenous metabolites. While dietary and membrane lipids, heme and metabolic waste products may not have originally

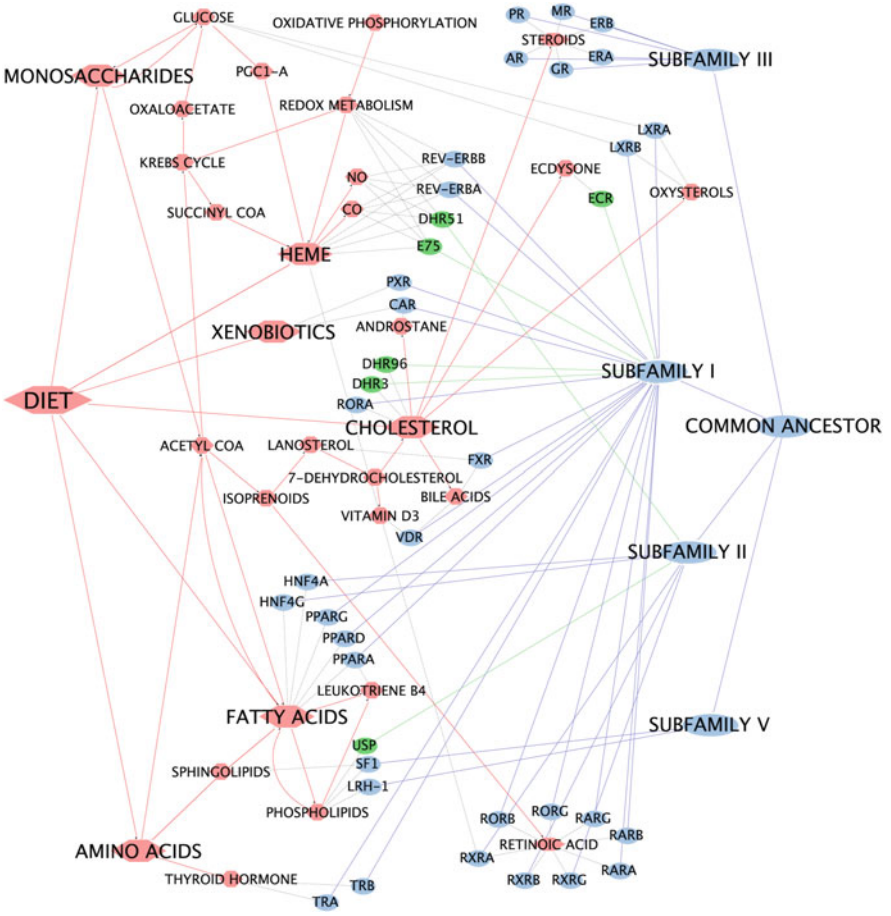

Fig. 6.5 Nuclear receptors form a network of sensors that synchronize target gene expression with diverse small molecule signaling and metabolic flux. In their surveillance of hormone signaling and disparate arms of metabolism, NRs integrate the chemical signaling environment of the cell with metabolic, developmental and reproductive gene expression. In a form of feedback regulation, many of these NRs regulate the genes involved in the regulation of their own ligands and other related metabolites. Examples of both receptor/ligand co-evolution (steroid receptors) and the independent evolution of receptor/ligand pairs (retinoids with RARs/RORs/RXRs) can be found in the superfamily. Originating with the dietary uptake, the metabolic pathways connect individual metabolites, marked by *red hexagons*. Nuclear receptors are indicated by *ovals*, which are either *blue* (human) or *green* (fly) and linked by phylogenetic relationships. Binding between ligands and receptors are indicated by *grey lines*. Orphan receptors that have yet to have cognate ligands identified are not shown

had the appeal of highly specific endocrine hormones, the importance of these ligands and their receptors has now been realized. These metabolic NRs are partly responsible for the now widely understood intercalation of metabolism with virtually all aspects of development and physiology [3, 94]. For example, recent work

has highlighted the potential role of metabolic NRs in the regulation of circadian rhythm and development [44, 105, 106]. In the fly, a network of NRs that are expressed in response to developmental pulses of ecdysone have been reported to bind and be transcriptionally regulated by a wide ranging collection of metabolites ([19, 21, 107–108]; DHR3: Krause unpublished results). The addition of metabolite sensing to the NR-mediated regulation of fly development brings a substantial layer of information-rich signaling to hormonal timing. These metabolic ligands have redefined the relationship between the NRs of the ecdysone response pathway from an autonomous system set in motion by an ecdysone pulse [109], to one that is responsive to the state of the organism and its environment.

Of the metabolic receptors, perhaps the fly receptor E75 and its human homologs, Rev-erbα and β, are the most novel. Until these receptors were recently de-orphaned, known NR ligands were limited to steroid hormones, fatty acids and other dietary and non-dietary lipids [3]. E75 and the Rev-erbs bind heme as a ligand and/or prosthetic group that allows for gas (NO, CO) and redox responsive transcriptional regulation [19, 22, 38, 39]. Like lipids, heme has long been recognized as an important molecule in metabolism. It is required for oxygen and carbon dioxide transport, for cytochrome function in the mitochondria and for the neutralization of reactive oxygen species (ROS) arising as a consequence of metabolism. It is also a required component of the cytochrome P450s that produce and break down most lipids, including those that serve as the ligands of most nuclear receptors [110–117].

In much the same way that E75, DHR3 and their ligands coordinate the developmental process of the fly, heme/gas/redox and cholesterol, respectively, serve as metabolic indicators to the mammalian molecular clock through the NRs Rev-erbα/β and RORα. As a regulatory couple, the Rev-erbs and RORα entrain the expression of other clock proteins to these fundamental measures of cell metabolism [22, 38, 39, 46, 118]. These inputs contribute to the more established modes of circadian entrainment, such as photoperiod, which together comprise a system of independent measures that coordinates metabolism with sleep wake cycles. This newly recognized capacity to monitor heme/gas/redox gives the NR superfamily access to regulatory signaling at the core of mammalian physiology. Heme abundance oscillates during the circadian cycle and, importantly, also functions as a prosthetic group to other circadian proteins, including NPAS2, Period2, and Clock [112–116, 119]. Given that heme is so central to respiration and other central metabolic processes, and that its abundance oscillates over time, it appears that heme serves as a fundamental measure of the diurnal metabolic state and as such provides feedback through the Rev-erbs and other clock proteins, to entrain the molecular clock to an organism's diurnal metabolic flux [22]. The circadian clock also appears to be in control of lunar and annual functions, which also need to be linked closely to nutrient availability and temperature fluctuations [120, 121].

Like heme, the other E75/Rev-erb regulators, redox and gas, are also generated in a circadian manner [117, 122, 123]. Redox homeostasis can be affected by the generation of reactive oxygen species (ROS), a large proportion of which arise not surprisingly from mitochondrial respiration. The redox state of a cell, or organelles,

is dependent on the ratio of ROS generated by metabolic activity and the abundance of antioxidants, both of which cycle diurnally (reviewed in [124–126]) and accordingly serve as an important measure of metabolic activity. Aside from the damage that ROS can cause, these molecules have also become recognized as important signaling molecules. Interestingly, ROS signaling is commonly associated with stress response [127], which like the molecular clock, is coordinated in the hypothalamus [128, 129]. Thus, as was mentioned earlier, distinct functions for NRs are often difficult to prescribe. In addition to a clear metabolic role, E75 and the Rev-erbs may also fulfill functions of stress-response, much like GR, or even xenobiotic surveillance of environmental oxidative stress.

As mentioned, the gases NO and CO also cycle with circadian periodicity. Heme is an essential component of both NO and CO producing enzymes, respectively Nitric oxide synthase and Heme oxygenase. Thus, not surprisingly, both NO and CO production have also been shown to oscillate diurnally [117, 122, 123]. The membrane permeable and transient nature of NO and CO gases conform to the ideal signaling properties of many other specialized NR ligands, and further connect the NR superfamily to a broad range of physiology.

It is interesting to note that, in retrospect, many of the processes influenced by Rev-erb proteins and their ROR counterparts, such as circadian rhythm, metabolism and inflammation, have long been known to involve NO/CO gas signaling [130]. For eample, there is an inverse relationship between heme and nitric oxide in the transactivation of NF-κB, a gene known to be regulated by Rev-erbα [131]. While heme leads to an increased activation of NF-κB [132], NO inhibits its activity [133, 134]. Likewise, NO and CO also affect the establishment and growth of cholesterol-rich plaques within arteries [135, 136], where RORs and Rev-erbs also play major roles [130, 137, 138]. This coincidence extends further to include mood/behavior disorders and obesity etiologies that have been associated with aberrant Rev-erb expression and/or circadian rhythm [139–143]. Taken together, one can imagine a scenario where the Rev-erb/heme/redox/gas signaling axis acts to coordinate overall energy management with diurnal cycles, feeding behaviour, local tissue metabolism and other related processes.

Our recent work in *Drosophila* shows that NO signaling via the Rev-erb/ROR orthologues, DHR3 and E75, also controls the timing of larval molts and metamorphosis, suggesting that transitions between growth, diapause and reproductive phases of the life cycle are also coordinated by these receptors (Caceres et al, in prep). Interestingly, disruption of these interactions results in either morbid obesity or wasting, depending on the direction of the genetic, ligand or chemical manipulation. The ability of these and other NRs to control and respond to dietary and circadian variations has likely played a major role in the ability of metazoa to adapt to so many diverse ecological niches. This assumption is consistent with the appearance of NRs during the Cambrian explosion and the ability of these new organisms to develop multicellularity and invade new ecosystems and environments [144]. Part of this diversification involved the evolution of new endocrine and metabolic organs that further enabled nutrient selection, uptake, storage and management, as well as efficient means of optimizing and balancing the growth and reproductive phases of

the lifecycle. Accordingly, recent evidence has shown that both stem cell pluripotency and differentiation into various cell and tissue types is also guided by the actions of a number of receptors [145–147].

As these new realms of ligand diversity and NR functions now show, the NR regulated processes of development, growth, metabolism and reproduction are deeply intertwined and reciprocally regulated, and it is the integration of these systems throughout the body that defines the NR superfamily. Also deeply related to these functions are the associated behaviors that make food consumption, reproduction and survival in different ecosystems possible. As with xenobiotic responses, the ability to mount immune responses to environmental pathogens is also under the control of NRs [94, 148, 149], and is modulated in a clock-dependent fashion [150].

In summary, it is clear that NR functions can no longer be categorized simply into hormonal or metabolic roles. A new subdivision into distinct functional categories is going to be challenging. This challenge will likely only grow as the roles of the less studied receptors, particularly the orphans, become better understood.

6.12 Medical Impact

Since Elwood Jensen's landmark discovery of the receptor for estrogen (ER), the degree to which NRs feature in the cause and prevention of diseases has become increasingly clear. ER on its own has been implicated as a major player in a broad range of disease states. As with the other early identified endocrine receptors, these include sexual, developmental and growth disorders, as well as a variety of cancers (reviewed in [151–157]). More recently, prominent roles for ER in obesity, behavioral disorders and aging have also been uncovered. Likewise, a survey of the literature reveals roles for most of the other NRs in virtually all aspects of human disease [4, 5, 158]. These diseases can be instigated by a variety of means including genetic mutations, endocrine tissue disruption, drugs and toxins, inappropriate diet, autoimmune disorders, lack of sunlight or the complexities of aging. For many of the same reasons that NRs and their ligands can cause disease, they can also play positive roles in disease prevention or cure. For example, Vitamin D and omega-3 fatty acids have recently been shown to have major beneficial effects on cancer prevention, immunity, metabolism, mood and memory.

Considering that many NRs control the expression of genes that promote cellular growth or differentiation, it is not surprising that NRs play a major role in cancer onset and progression, as well as prevention and therapy. A classic example is the role of ERs in breast cancer, and the use of antagonists such as tamoxifen or raloxifene to treat it. Similarly, many other NRs have since been linked to the onset, progression and treatment of many other cancers (reviewed in [159]). Recent examples of NRs and ligands being used to treat or prevent cancer include VDR, RAR, RXR and their cognate ligands to reestablish programmed cell death in various tumor types [160–162].

NRs play a major role in diseases stemming from defects in immunity. These include a large variety of autoimmune diseases, asthma, acnes, and numerous other

inflammatory reactions. Since its discovery in 1948, the GR ligand cortisone has been used to treat many of these diseases and reactions [159, 163–165]. More recently, selective GR agonists (SEGRAs) have garnered considerable attention as potential therapeutics in the treatment of autoimmunity [166]. In addition to GR, several other NRs have been shown to be involved in the regulation of immune responses (reviewed in [165]). For example, ER has been implicated as a potential target in regulating autoimmune responses that underlie multiple sclerosis [167], and FXR, PXR and VDR, originally characterized for their roles as bile acid and xenobiotic sensors, have emerged as potent modulators of immune and inflammatory reactions in entero-hepatic tissues (reviewed in [168]). PPARγ, LXRα and β, VDR, NURR1, and RAR have also now been shown to have important regulatory functions in immune cells (reviewed in [165, 169, 170]), with PPARγ recently shown to also play a prominent role in multiple sclerosis (reviewed in [171]).

Considering their roles as core components of metabolic homeostasis, it is not surprising that NRs contribute significantly to metabolic diseases. The fundamental importance of these regulatory networks is becoming increasingly clear in light of the rapidly rising, near-epidemic levels of metabolic disorders that comprise "metabolic syndrome". These include obesity, diabetes, cardiovascular disease, hyperlipidemia, atherosclerosis and hypertension [3, 4]. The following projections from the World Health Organization (WHO) provide some insight into the frequency of metabolic disorders worldwide [172].

- Globally in 2005 approximately 1.6 billion adults (age 15+) were overweight and at least 400 million adults were obese.
- In 2005, an estimated 1.1 million people died from diabetes.
- More than 220 million people currently have diabetes.
- By 2015, approximately 2.3 billion adults are expected to be overweight and more than 700 million obese.

Although the most sensible long-term solution to this problem lies in prevention, molecular medicine has shown tremendous promise in offering a means of treatment in the late stages of these diseases, and in extreme cases where dietary modification on its own is insufficient to restore health.

Although it is clear that the types and volumes of food currently consumed in modern societies are a major contributor to the current metabolic disease pandemic, it appears that a number of industry generated (synthetic?) compounds may also be to blame. It has been known for some time that industrial compounds such as Bisphenol A (BPA), and contraceptive contamination of wastewater runoffs, affect ER and ERR activities in animals and humans. However, it is becoming increasingly clear that these and other endocrine disrupting compounds also affect a number of other NRs, including GR, TR, PPARs and RXRs, with striking effects on adipocyte proliferation, differentiation and function (reviewed in [173–175]). Several new screening strategies capable of identifying these NR-targeted "obesogens" have recently been described [176–178]. These approaches hold great promise towards

the identification of obesogenic compounds in industrial, agricultural and municipal effluents and byproducts.

Another emerging area in which NRs have been shown to play a critical role is circadian rhythm and associated sleep-based disorders. As mentioned previously, the role of the Rev-erbs and the RORs in the mammalian circadian clock has become increasingly evident. The identification of several other NRs including ER, RAR, PPARα and γ, and EAR2 as regulators of the circadian clock has helped to further demonstrate that NR signaling and metabolism form an integral part of the circadian timing system (reviewed in [179–183]). Considering the importance of circadian rhythm in the regulation of metabolism, obesity and depression, it will be important to fully explore the medical implications of these circuits and mechanisms.

Circadian and metabolic clocks also play a key role in controlling lifespan. Without exception, excessive dietary intake leads to life threatening diseases, while reduced caloric intake has been shown to prolong life span [184–189]. Cholesterol, lipid metabolism and NRs have also been linked to a variety of other age-associated neuronal diseases such as Alzheimer's, Parkinson's, Niemann Pick, Fragile X and Huntington disease (reviewed in [21, 190–194]). Correspondingly, several NRs have been identified as playing particularly important roles in related neuronal processes. Examples include ER, which has been shown to regulate cognition and synaptic plasticity [195, 196], LXR, which functions as a major regulator of genes involved in cholesterol homeostasis and which has been implicated in Alzheimer's [197], the PPARs, which function as regulators of aging through their roles in lipid homeostasis [198, 199], and VDR which elicits neuroprotective functions and plays a beneficial role in both the developing brain and in adult cognition [200, 201]).

Considering the well-established interplay between circadian rhythm, metabolism, cancer, and immunity it will be important to further understand how NRs regulate and integrate these superficially distinct processes. Further NR ligand identification should help provide new insights into the pathways and processes that give rise to these diseases, as well as new means to prevent and treat them.

6.13 Conclusions

It seems increasingly clear from research in model organisms, and with the latest round of ligand discoveries, that the general role of NRs is to match rates of growth, development, and reproduction to the available dietary and physical offerings provided by unique environmental niches. The co-diversification of NR proteins and ligands has allowed metazoa to adapt and differentiate their lifecycles, diets, metabolism and behaviors to meet the challenges of diverse and hostile environments.

While the importance of NRs in mammalian physiology and disease has helped spur considerable research and progress, we still know relatively little about the majority of NRs outside this metazoan class. There is still much to learn about all of the existing orphans, and about the roles of NRs in numerous tissues and developmental stages. One particularly challenging frontier will be the brain, where both

NRs and their ligands are particularly abundant. Behaviors linked to metabolism, development, sexual diversification and reproduction will likely be controlled by these NRs and their metabolites.

Although a growing body of research is finding that NRs are subject to many forms of ligand-independent regulation, which play important roles in controlling and fine-tuning NR activities and functions, it will likely continue to be the discovery of new ligands that drives this field forward at a maximal pace. These ligands will switch on the lights that illuminate new and unexpected roles and pathways.

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