

**Research Article** 

# **Conserved and Exapted Functions of Nuclear Receptors in Animal Development**

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**Abstract.** The nuclear receptor gene family includes 18 members that are broadly conserved among multiple disparate animal phyla, indicating that they trace their evolutionary origins to the time at which animal life arose. Typical nuclear receptors contain two major domains: a DNA-binding domain and a C-terminal domain that may bind a lipophilic hormone. Many of these nuclear receptors play varied roles in animal development, including coordination of life cycle events and cellular differentiation. The well-studied genetic model systems of *Drosophila*, *C. elegans*, and mouse permit an evaluation of the extent to which nuclear receptor function in development is conserved or exapted (repurposed) over animal evolution. While there are some specific examples of conserved functions and pathways, there are many clear examples of exaptation. Overall, the evolutionary theme of exaptation appears to be favored over strict functional conservation. Despite strong conservation of DNA-binding domain sequences and activity, the nuclear receptors prove to be highly-flexible regulators of animal development.

Keywords: development, C. elegans, Drosophila, mouse, functional conservation, exaptation.

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Among the earliest nuclear receptors (NRs) to be defined by mutation are androgen receptor in mice in 1970 [1], *unc-55/COUP-TF* in *C. elegans* in 1973 [2], *knirps* in *Drosophila* in 1980 [3], and *daf-12* in *C. elegans* in 1981 [4]. While their molecular identity and characterization as NRs largely required the development of positional cloning strategies of the 1980's, there is a long record of genetic analysis of NRs in model systems. Most of these early mutations were recovered from unbiased forward genetic screens for strong developmental phenotypes, highlighting the importance of NRs as regulators of ontogenetic mechanisms.

The typical nuclear receptor includes two major domains: a DNA-binding domain (DBD) that allows sequence-specific binding to DNA, and a ligand-binding domain (LBD) that binds a lipophilic hormone in some cases. Both ligand-dependent and ligand-independent NRs function as important regulators of transcription. They arose over 500 million years ago, apparently concomitant with the genesis of animal life. NRs are represented in all animal phyla, including Porifera, but are not found in plants or prokaryotes [5–7]. Thus the NRs are included in the basic genetic "toolkit" that animals adapted and refined over the course of evolution [8]. Several lines of evidence argue that lipophilic hormone binding activity—which makes them so interesting from the perspective of endocrinology and human health—is a derived property [6, 9, 10]. Indeed, some of the earliest NR genes defined by mutation produce proteins that are believed to be ligand-independent, such as *unc-55* and *knirps* [2, 3]. This suggests that the ancestral function of NRs was DNA-binding and transcriptional regulation.

In various phyla, NRs have proved to be a nimble and adaptable gene family. There are multiple examples of gene loss in specific animal clades, as well as massive gene proliferation and divergence in specific clades, such as the radiation of HNF4 NRs in C. elegans [6, 11]. Many species have evolved atypical nuclear receptors, including some that lack a DBD or LBD, or have two DBD's [5, 12]. An example of this includes the 2DBD-NRs in the flatworm Schistosoma mansoni, which have two DBD's in tandem with one LBD [12]. Some of the first geneticallydefined NRs were discovered in the classic *Drosophila* patterning screens [13]. However, NRs show less functional conservation in development than some of the homeobox genes, which include conserved arbiters of axis positional information in vertebrates and invertebrates [13]. Despite the long history of research on NRs as regulators of developmental processes, the majority of published work has focused on vertebrate-specific steroid receptors [7]. In this review, we take a broad biological view of the genetic function of conserved NRs in development, with an eye towards patterns of exaptation (repurposing; [14]) and functional conservation across phylogeny. Because an understanding of developmental mechanisms benefits from analysis of organisms with robust genetic technologies, we will focus primarily on orthologs from the animal model systems Drosophila, C. elegans, and mouse.

Genome sequencing projects have allowed a fairly comprehensive analysis of the conservation and divergence of nuclear receptors among different phyla. The recent completion and annotation of mollusk, annelid, and platyhelminth genomes has provided additional clarity on patterns of conservation and orthology [12, 15, 16]. Including lophotrochozoan NR proteins produces a phylogenetic analysis that is largely consistent with standard nomenclature for nuclear receptors (Figure 1). Each systematically-numbered NR class (e.g. NR2B, NR4A [6]) generally assembles nicely into a well-supported clade with orthologs in various species. Bootstrap replicates, as a test of the reliability of each clade, are strong for well-conserved nuclear receptors such as the various NR2 members (ranging from 58% support for NR2E1 to 93% support for the NR2F clade). In contrast, some NR1 clades assemble relatively poorly, with lower bootstrap support of reliability, consistent with the previously-noted sequence divergence of these NRs [6]. Short branch lengths illustrate the strong sequence conservation of DBD sequences over long evolutionary periods.

We identified 18 nuclear receptor genes that are represented in all three major phyletic groups (Ecdysozoa, Lophotrochozoa, and Deuterostomata) and therefore show clear ancient evolutionary origins: one each from NR class 1D, 1E, 1F, 1H, 1J, 2A, 2B, 2C, 2F, 3B, 4A, 5A, 5B, and 6A, and four from NR class 2E (Table 1; Figure 1). Eleven of these also have apparent orthologs in the platyhelminth *S. mansoni* [12], providing further evidence for their Urbilaterian origin. Four have been lost from Chordata, but are represented in Ecdysozoa and Echinodermata (NR1E/E78, NR2E4/DSF, NR2E5/HR83, and NR5B/HR39). Four additional nuclear receptors show more complicated patterns of conservation, being absent from Ecdysozoa, but present in Lophotrochozoa and Deuterostomata (NR1A/TRa, NR1B/RAR, NR1C/PPAR, and NR3A/ER). This could result from gene loss in early ecdysozoan evolution or the generation of novel NR genes in a common ancestor to lophotrochozoans and deuterostomes. The presence of an apparent NR1A/TR ortholog in *S. mansoni* argues for the former interpretation for that gene, although it is possible that this represents an example of horizontal gene transfer from chordates. Because these four nuclear receptor subfamilies are not represented in Ecdysozoa, they are not considered further. For the purposes of this review, we focus our analysis on the 18 nuclear receptors

Platyhelminthes Ecdysozoa			Lophotrochozoa	Echinodermata	Chordata	
Class	S. mansoni	D. melanogaster	C. elegans	C. gigas	S. purpuratus	H. sapiens
1A	TRa/TRb			NR1A	THR	$TR\alpha/\beta^1$
1B				NR1B	RAR	RAR $\alpha/\beta/\gamma^1$
1C				NR1C/CDa/CDb	PPAR 1/2	PPAR $\alpha/\beta/\gamma^1$
1D		E75	NHR-85	NR1D	RevErb	Rev-Erb $\alpha/\beta^1$
1E	E78	E78	SEX-1	NR1E	E78 a/b	
1F		HR3 <sup>1</sup>	NHR-23	NR1F		ROR $\alpha/\beta/\gamma^1$
1H		$EcR^1$	(Brugia EcR)	NR1H	FXR	LXR $\alpha/\beta^1$
						$FXR^1$
1J	HR96	HR96	DAF-12 <sup>1</sup>	NR1J a/b/g		$PXR^{1}$
			NHR-8/-48			VDR <sup>1</sup>
						$CAR^1$
2A	HNF4	HNF4	superfamily	NR2A	HNF4	HNF4 $\alpha/\gamma^1$
2В	RXR1	USP	(Brugia USP)	NR2B	RXR	RXR $\alpha/\beta/\gamma^1$
	RXR2					
2C	TR2/4	HR78	NHR-41	NR2CD	NR2C	TR2
					TR2/4	TR4
2E	TLL	TLL	NHR-67	NR2E1	TLX	TLX
2E	DSF	DSF		NR2E4	NR2E4	
2E	PNR	UNF	FAX-1	NR2E3	PNR	PNR
2E		HR83	NHR-239	NR2E5	NR2E5	
2F	COUP-TFI	SVP	UNC-55	NR2F	COUP-TF	COUP-TF α/β
	COUP-TFII					EAR2
3A				NR3A		ER $\alpha/\beta^1$
3B		ERR		NR3B	ERR	ERR $\alpha/\beta/\gamma$
4A	NR4A5	HR38	NHR-6	NR4A	NURR	Nur77
						Nurr1
						NOR1
5A	FTZ-F1a	FTZ-F1 <sup>1</sup>	NHR-25/-97/	NR5A	LRH-1	SF-1 <sup>1</sup>
			-119			LRH-1 <sup>1</sup>
5B	FTZ-F1b	HR39 <sup>1</sup>		NR5B	NR5B	
6A		HR4 <sup>1</sup>	NHR-91	(Aplysia NR6A)	GCNF	GCNF
					GRF	

 Table 1: Conserved nuclear receptors.

<sup>1</sup>NR with well-defined ligand.

Shaded rows identify the 18 NRs with broad phylogenetic conservation discussed in this review.

(shaded gray in Table 1) that display broad polyphyletic conservation and are thus represented in the classic developmental model system of *Drosophila*.



Figure 1: (preceding page). Molecular Phylogenetic Analysis of Nuclear Receptors. We generated a phylogenetic tree using the Maximum Likelihood method based on the Dayhoff matrix based model [266] from amino acid sequences of selected conserved NRs. Sequences were obtained from the NCBI database, aligned using Clustal W, and trimmed to include only the extended DBD for each sequence record (this is helpful when including C. elegans NRs, which have highly-diverged LBDs [11, 185]). The tree with the highest log likelihood (-10026.43) is shown. The small number placed at each branch indicates the percentage of trees in which the associated taxa clustered together in 100 bootstrap replicates. Initial tree for the heuristic search was obtained by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted to the atypical (NR0) nuclear receptor knirps as an outgroup. Analysis was conducted using MEGA7 [267]. For each class, a C. elegans (or Brugia malayi), Drosophila, Mus musculus, and Danio rerio ortholog was included (when present), organizing the analysis around two ecydysozoans and two vertebrates. Additional NRs were added from the complete genome of the echinoderm S. purpuratus, and two lophotrochozoan species. The lophotrochozoan species included varied due to the differences in completeness and annotation of these genomes, as well as a few possible cases of gene loss. Whenever possible, a representative annelid and mollusk was included. Species abbreviations: Dm Drosophila melanogaster, Ce Caenorhabditis elegans, Dr Danio rerio, Mm Mus musculus, Bm Brugia malayi, Sp Strongylocentrotus purparatis, Ac Aplysia californica, Cg Crassostrea gigas, Ct Capitella teleta, Hr Helobdella robusta, and Lg Lottia gigantea. This phylogenetic analysis establishes relationships among the major conserved nuclear receptor that is largely consistent with previouslypublished studies (e.g., [6]), but includes new data from the more recently-completed invertebrate genomes.

#### 1. E75/NHR-85/Rev-Erb (NR1D)

The *Drosophila E75* gene functions in molting, metamorphosis, oogenesis, and circadian rhythms [17, 18]. It is essential for larval development, responding to ecdysone regulatory pathways at the onset of metamorphosis [19]. *E75* is required for ecdysteroid biosynthesis in the prothoracic gland, and it counteracts *DHR3* to avoid the premature repression of steroidogenesis [20]. Germline clones of *E75* mutations cause germline arrest prior to meiosis entry, and degeneration of oocytes with follicle cell migration defects [21, 22]. In *Drosophila* males, *E75* promotes stem cell maintenance in the testis, and knockdown causes a loss of germ stem cells and cyst stem cells [23]. The final known role of *E75* in *Drosophila* is similar to that of its mammalian ortholog, Rev-Erb: *E75* functions in the fly molecular clock and circadian rhythm in clock and pacemaker neurons [24]. *E75* and *unf* (see below) act together to enhance transcription of *per*, which finishes the transcriptional feedback loop needed for rhythmic cycles of gene expression within cells [19].

The *nhr-85* gene in *C. elegans* is less well-characterized. RNAi knockdown experiments indicate that *nhr-85* plays a role in the development of the egg laying system, vulval development, and formation of a proper dauer larva cuticle [25]. Like its *Drosophila* homolog *E75*, *nhr-85* functions in both cuticular development and female organogenesis. The gene appears to be upregulated at larval molts. *C. elegans* molts punctuate periods of quiescent inactivity that represent a sleep-like state [26]. Therefore, upregulation of *nhr-85* in anticipation of quiescent periods may be functionally analogous to circadian function in flies.

The vertebrate homologs, Rev-Erb $\alpha$  (NR1D1) and Rev-Erb $\beta$  (NR1D2), play a significant role in circadian rhythms and metabolism [27, 28]. Rev-Erb paralogs are expressed in neurons, the thymus, skeletal muscle, and kidney [29]. Mutant mice lacking both Rev-Erb paralogs in the liver have arrhythmic expression of the clock genes Bmal1 and Cry1 [30], reminiscent of *E75* function in the fly. In the zebrafish *Danio rerio*, Rev-Erb $\alpha$  is similarly important for circadian control of autophagy rhythms [31, 32]. A double knockout of both Rev-Erb genes in mice yields animals that are fairly normal and viable, suggesting that the genes do not play a major role in development. However, cerebellar defects have been described in Rev-Erb $\alpha$  knockout mice, leaving open the possibility of a developmental function [33]. Neither Rev-Erb gene is known to play a role in reproductive development.

In summary, the NR1D gene family appears to have a broadly conserved function in controlling cellular rhythms across animal phyla, particularly in control of arousal. Developmental functions are evident in both flies and worms; however conservation of the specific functions is ambiguous at best. These functions may represent separate examples of exaptation.

# 2. E78/SEX-1 (NR1E)

In *Drosophila*, *E78* functions in female reproductive development and is ecdysone-induced. Loss of *E78* results in decreased numbers of cap cells, germline stem cells, and follicle cells [34, 35]. As a consequence *E78* mutants lay few eggs and display early onset female sterility [34]. Embryos from *E78* mothers fail to complete gastrulation. Finally, like *E75*, *Drosophila E78* is required for ecdysone regulatory pathways that control the onset of metamorphosis [19].

The *C. elegans* homolog of *Drosophila E78* is *sex-1*, which plays a role in the regulation of nematode sex [36, 37]. The *xol-1* gene specifies male development, and *sex-1* is responsible for repressing *xol-1* transcription, leading to hermaphrodite sexual development. Mutations in *sex-1* cause death from multiple developmental defects due to inappropriate dosage compensation [36].

While both nuclear receptor genes function in sex-specific development, the roles of these genes differ across species. *E78* functions in *Drosophila* female reproductive development, whereas *sex-1* plays an early key role in global sex determination of *C. elegans* in multiple tissues. *E78* also has an early embryonic function unrelated to sex-specific development, which has not been described for *sex-1*. Thus even though there are hints at some common, conserved function, this class also illustrates the theme of exaptation. Although bona fide *E78* orthologs appear to be present in the platyhelminth *S. mansoni*, lophotrochozoans, and echinoderms, the gene appears to have been lost in vertebrate lineages (Table 1).

# 3. HR3/NHR-23/ROR (NR1F)

The *Drosophila HR3* gene was identified by molecular strategies on the basis of sequence relationship to E75 [38]. It is ecdysone-responsive and initially serves to repress other ecdysone-responsive genes that trigger metamorphosis and then induces expression of *FTZ-F1* as part of the prepupal response to ecdysone [39, 40]. Deletion of *HR3* results in embryonic lethality with severe ventral nerve cord and tracheal defects, indicating that it plays a central role in development [41, 42]. Mutants that are permitted to reach larval stages expire at morphogenesis with multiple defects in wing, bristle, and cuticle formation [39]. Therefore, *HR3* is an important developmental control gene with various roles in patterning embryos as well as temporal coordination of life cycle transitions.

The *C. elegans HR3* ortholog, *nhr-23*, plays an apparently similar role in coordinating molting and developmental transitions. While embryonic functions appear likely, they have not been evaluated in detail. Mutations in *nhr-23* cause mid-larval arrest with cuticle defects, demonstrating a critical function in hypodermal development and cuticle formation [43, 44]. Expression

of the gene is sharply up-regulated with larval molts [25], and it is a target of the heterochronic miRNA *let-7* that regulates transitions between larval stages [45].

The three RAR-related orphan receptors (ROR; NR1F1, NR1F2, NR1F3) of vertebrates were first identified by molecular strategies in screens for new nuclear receptors [46–49]. Mutations in ROR $\alpha$  were found to be the cause of the *staggerer* mutation in mice [50]. These mice present with severe cerebellar dysfunction and altered gait, due to an underlying defect in proliferation and differentiation of Purkinje cells [50, 51]. Like its invertebrate orthologs, ROR $\alpha$  is a critical regulator of multiple developmental events [52–54]. Metabolic and immunological functions have been described [53, 54], as well as a role in circadian gene regulatory patterns [55]. The ROR $\beta$  gene is expressed in a circadian pattern in the eye and pineal gland. Deletion of ROR $\beta$ in mice results in altered gait, suggestive of underlying cerebellar dysfunction, disorganized retinas with defective rod photoreceptors, and progressive blindness [56, 57]. Recent studies have suggested a broader role in cerebral neuron development, with loss of ROR $\beta$  associated with epilepsy and intellectual disability in humans [58]. Deletion of the third paralog, ROR $\gamma$ , results in defects in the production of thymic cells and lymph nodes, due to a critical role for this nuclear receptor in maintaining these cells and preventing apoptosis [59]. Mutations in the human ROR $\gamma$  gene cause immunodeficiency [60].

The common function in life cycle transition in both *Drosophila* and *C. elegans* illustrates some level of functional conservation for the NR1F class. While not exactly the same, the vertebrate functions in circadian rhythms are at least reminiscent of the broad, temporal functions of the gene class in Ecdysozoa. The repurposing of vertebrate genes to immune system development is a clear case of exaptation.

#### 4. EcR/LXR/FXR (NR1H)

The steroid hormone 20-hydroxyecdysone is produced in the prothoracic glands and serves as the primary endocrine signaling system that coordinates life cycle transitions in the fly [61]. Ecydsone receptor (*EcR*) mediates the hormonal response in various tissues [62] via heterodimers with the coordinating nuclear receptor *usp* (NR2B; described below). *EcR* functions in germ band retraction and head involution during morphogenesis of the embryo [63]. *EcR* and *usp* play a central role in late third instar larvae in responding to the ecdysone pulse that triggers pupation and metamorphosis. In mutant larvae, imaginal discs fail to expand and differentiate, the larval gut and salivary glands fail to undergo apoptosis, and the adult gut does not form. In the developing wing, sensory neurons of *usp* and *EcR* mutant larvae undergo precocious differentiation [64]. *EcR* and *usp* also function in the development of male-specific neuronal architecture and male-mating behaviors [65]. More recently, *usp* and *EcR* have been shown to be required for the survival of prothoracic gland cells during metamorphosis via an ecdysone-independent pathway [66].

Surprisingly, *C. elegans* lacks both an *EcR* and an *usp* ortholog [25]. Despite ecdysis as a central property of Ecdysozoa, *Caenorhabditis* appears to have dispatched this pathway entirely. Nonetheless, several nuclear receptors, in particular *nhr-23* and *nhr-25*, perform molting regulatory functions in *C. elegans*, providing an example of the flexibility of nuclear receptors, even in broad physiological and developmental control. It is important to note that this is not a feature of nematodes in general, since the parasitic nematode *Brugia malayi* and free-living

nematode *Pristionchus pacificus* both retain EcR, RXR, and ecdysteroid-based regulation [67, 68]. Instead, this unexpected gene loss appears to be a peculiarity of an evolutionary lineage within the Rhabdita order.

Two vertebrate orthologs of EcR, LXR $\alpha$  and LXR $\beta$  (Liver X Receptor; NR1H3 and NR1H2, respectively), were initially identified in several labs using molecular techniques [69, 70]. Just like EcR and USP, both LXRs heterodimerize with RXR partners (NR2B; see below) and are responsive to oxysterol ligands [71]. Mice deleted for LXR $\alpha$  display defects in cholesterol metabolism and inflammatory responses, but were born viable and healthy, suggesting that developmental functions are few or redundant [69]. LXR<sup>β</sup> mutations lead to adult-onset motor neuron degeneration in male mice [72], inflammatory response defects, and hyperplasia of Tcells [73]. These effects appear to be secondary to cholesterol metabolism defects rather than direct functions in development. Loss of both LXR $\alpha$  and LXR $\beta$  results in susceptibility to bacterial infection due to apoptosis of macrophages, indicating that LXR function is required for macrophage survival, but not generation [74]. A third vertebrate-specific paralog of the LXR's is FXR (Farnesoid X Receptor; NR1H4). This gene was identified by molecular strategies from a rat liver cDNA library and shown to bind bile acids [75]. Knockout of the gene in mice results in animals that are apparently developmentally normal, but display elevated bile acid levels and metabolic defects [76]. Therefore, the broad developmental regulatory functions evident in Drosophila appear to have been dropped in vertebrate lineages.

### 5. HR96/DAF-12/VDR/PXR/CAR (NR1IJ)

The *HR96* gene of *Drosophila* was first identified by molecular strategies [19]. Mutations in the *HR96* gene cause defects in triacyl glycerol and cholesterol metabolism, as well as increased sensitivity to xenobiotics such as phenobarbital and the insecticide DDT [77–81]. Research on *HR96* has served as a model to understand cholesterol metabolism and detoxification [82, 83]. No significant role in regulating developmental events is evident.

The daf-12 nuclear receptor is the most extensively-studied NR in C. elegans due to its pivotal role in regulating response to TGF $\beta$  and insulin signaling in the dauer developmental pathway and regulation of lifespan [84]. The dauer is a long-lived, highly-resistant dispersal larva form of the nematode that is an alternative to the normal L3 larval stage [85]. Mutations in daf-12 were first identified in screens for defective formation of dauer larvae [4]. Subsequent research showed that daf-12 encoded a Vitamin D Receptor-related gene that binds steroid hormones called dafachronic acids [86, 87]. The gene broadly regulates temporal developmental and metabolic processes in the worm [84]. In the presence of food and lower populations of worms, the enzymatic product of the *daf-9* gene produces dafachronic acids that activate the DAF-12 nuclear receptor, prompting it to activate transcription of target genes that favor normal reproductive development. When food becomes scarce and population conditions more crowded, DAF-12 binds the co-repressor DIN-1, shutting off its normal target genes and promoting a developmental switch to dauer larvae. The daf-12 gene also regulates heterochronic miRNA genes that are responsible for the timing of earlier developmental transitions [88], thus serving multiple roles in coordinating development in multiple tissues. Interestingly, the functions of daf-12 appear to be more conserved with *EcR* than its apparent fly and vertebrate orthologs, all of which function in lipid metabolism. In both flies and insects, insulin signaling and TGF $\beta$  signaling

converge with the production of a lipophilic hormone that binds EcR (in the fly) or DAF-12 (in the worm), both of which regulate the *let-7* miRNA that in turn mediates major life cycle transitions [89, 90]. Interestingly, this pathway may also have a vertebrate analog, whereby estrogen signaling activates the transition to puberty via Estrogen Receptor. Another *daf-12*-like paralog in *C. elegans*, *nhr-8*, is expressed in the gut where it regulates cholesterol balance, fatty acid desaturation, apolipoprotein production, and bile acid metabolism, similar to the function of *HR96* in flies, suggesting that duplication of this gene class in nematodes may have allowed segregation of functions [91]. An additional paralog, *nhr-48*, has no obvious phenotype when deleted and has not been extensively characterized.

Vertebrates possess three genes with apparent relationship to the *HR96/daf-12* class: Vitamin D Receptor (VDR; NR1I1), Pregnane X Receptor (PXR; NR1I2), and Constitutive Androstane Receptor (CAR; NR1I3/4). All three were initially identified using molecular approaches [92–94]. Mouse knockouts of VDR are born overtly normal, but die within 15 weeks of birth with significant deficits associated with Vitamin-D deficiency, such as rickets [95]. In addition, female animals suffer from uterine and follicle developmental defects, which are not observed with Vitamin-D deficiency, raising the possibility for non-Vitamin-D dependent developmental functions for VDR. Subsequent work showed that some bone defects were caused by a failure of apoptosis in proliferating chondrocytes [96], and that natural killer T cells also depend on VDR for their proper development [97].

The PXR gene (aka SXR in humans) activates cytochrome P450-3A expression in response to xenobiotics and plays a critical role in mediating drug responses [98–100]. Similarly, the CAR gene functions in xenobiotic response by upregulating the cytochrome P-450 Cyp2b10 gene [101–103]. Mice lacking the CAR gene are developmentally normal and fertile [102].

The functions of PXR and CAR appear to conserve the function of *Drosophila* HR96 in at least some respects, and the NR1I and NR1J subfamilies seem to play broadly-conserved roles in metabolic regulation. The developmental function of *daf-12* in *C. elegans* appears to be a distinct exaptation, although it is worth noting that dauer formation is a developmental event that is intimately linked to metabolic regulation.

#### 6. HNF4 (NR2A)

The *HNF4* gene is well-conserved across animal evolution. The *Drosophila HNF4* gene was identified by molecular techniques on the basis of its similarity to the vertebrate gene [104]. Early analysis of expression patterns and a chromosomal deletion suggested that *HNF4* might play a role in fly gastrulation and gut development as in vertebrates [104]. However, *HNF4* null mutants hatch normally, indicating that the gene is not essential for gastrulation. Mutant larvae display metabolic defects in utilizing stored fat during larval life before arresting development either just before or after pupal eclosion [105]. Thus fly *HNF4* appears to have a major role in regulating metabolism, but a less well-defined role in development.

The HNF4 class of nuclear receptor has undergone a remarkable expansion in *C. elegans* and related nematodes to generate hundreds of distinct family members (269 distinct paralogs in *C. elegans* [106]). While most of these nuclear receptors have not yet been functionally-characterized, of the dozen or so that have been studied most play some role in regulating metabolism, similar to flies and vertebrates [90]. This includes the well-characterized nuclear

receptors *nhr-13*, *-49*, *-63*, *-64*, *-66*, *-69*, *-76*, and *-80*. In particular, the function of *nhr-49* in regulating fat metabolism has striking parallels to the function of *Drosophila* HNF4 in the same process [107, 108]. One possibility is that multiple metabolic functions of fly and vertebrate HNF4 genes have been distributed to various individual *C. elegans* HNF4 family members [109]. Some *C. elegans* HNF4 nuclear receptors have taken on key functions in development as well. The *nhr-31* gene functions in the growth of the excretory tube [110], *nhr-114* functions in somatic tissue to regulate germ-line proliferation in response to diet [111], *nhr-40* functions in embryonic elongation and muscle development [112], and *nhr-60* functions in hypodermal cell differentiation [113]. The HNF4 class has apparently expanded functionally to incorporate a range of physiological and developmental functions.

The first HNF4 ortholog was identified in vertebrates from liver extracts using molecular approaches [114]. Mice and humans have two paralogs, HNF4 $\alpha$  and HNF4 $\gamma$  (NR2A1; NR2A3). Deletion of the HNF4 $\alpha$  results in failure of late stages in gastrulation, indicating that the gene plays a central role in the development of the gut [115–117]. Analysis of gene function identified an important role in insulin production in the pancreas [118], and in hepatocyte differentiation [119]. Therefore, metabolic defects in HNF4 mutants may reflect secondary consequences of underlying developmental defects.

A recent study has also implicated HNF4 $\alpha$  in the development of neural stem cells, hinting at broader developmental functions for the gene class in vertebrates [120]. The HNF4 $\gamma$  paralog has not been genetically-characterized, but expression analysis indicates that it could play roles in multiple organs [121]. A third paralog (HNF4 $\beta$ /NR2A2) has been identified in *Xenopus* [122].

Overall, functions of the HNF4 class have concentrated largely on metabolic regulation in Ecdysozoa, but this is not a conserved function in vertebrates. Given the large number of HNF4-related genes in *C. elegans*, it is difficult to make direct comparisons, but at least some have functions in the differentiation of cells and organogenesis. However, none of the functions identified so far align well with known vertebrate functions in development.

### 7. USP/RXR (NR2B)

The *ultraspiracle* gene of *Drosophila* was first identified as a recessive embryonic lethal mutation [123]. Subsequent molecular work identified it as an RXR ortholog that served as a heterodimerization partner 16 for EcR, and thus *usp* is a critical mediator of ecdysone signaling in flies [124, 125]. The major functions of *usp* are largely covered above under the functions for its partner, *EcR*. However, *usp* and *EcR* mutants have somewhat different embryonic phenotypes, suggesting that *EcR* has additional functions that do not depend on *usp* [123]. Nonetheless, as is the case for *EcR*, *usp* function is required in multiple tissues for cell survival and differentiation, consistent with its role as the cofactor for a global integrated hormonal response. As described above, *C. elegans* lacks an NR2B ortholog, just as it lacks an *EcR* ortholog.

The retinoid-binding RXR receptors were first identified in 1989 on the basis of molecular properties in vertebrates [126–128]. The three paralogs (RXR $\alpha$ , RXR $\beta$ , RXR $\gamma$ ; aka NR2B1, NR2B2, NR2B3) serve as key heterodimer partners for various NR1 nuclear receptors, including retinoic acid receptors, thyroid hormone receptors, PPARs, and LXRs, the vertebrate ortholog of fly *usp* partner *EcR*, demonstrating both conservation and flexible elaboration of their functional properties over evolution [7]. Knockout of RXR $\alpha$  in mice results in embryonic arrest with severe

hypoplasia of the heart and eye malformations, identifying this receptor as a key regulator of retinoid signaling in cardiac and ocular organogenesis [129, 130]. Knockout of RXR $\beta$  results in some embryonic lethality without a clear cause. Surviving animals are mostly normal, but males suffer from defects in spermatogenesis due to abnormalities in Sertoli cells [131]. Similarly, mice lacking the RXR $\gamma$  gene are viable, normal in appearance, and fertile [132]. This is not a simple matter of redundancy among the RXR paralogs, since RXR $\alpha^{+/-}$  RXR $\beta^{-/-}$  RXR $\gamma^{-/-}$  triple mutants are also viable and crudely normal. More detailed analysis has revealed that RXR $\beta$  and RXR $\gamma$  do play a role in neuron development or physiology, since knockout mice suffer from memory and affective behavior deficits [133, 134]. Studies have also identified roles for RXR receptors in cyclic metabolic functions in the liver and vasculature [135, 136], suggesting some conservation of global temporal regulation reminiscent to *usp* function in the fly. Nonetheless, despite the apparent conservation of heterodimerization properties, this subfamily also demonstrates significant functional elaboration across animal phyla.

# 8. HR78/NHR-41/TR2/TR4 (NR2CD)

The *Drosophila HR78* gene was identified by molecular approaches as a nuclear receptor expressed during the onset of metamorphosis [19]. Its expression is induced by ecdysone and oscillates with the molting cycle, but it functions as a negative regulator of EcR/USP activity. Loss of *HR78* results in arrest as undersized third instar larvae that fail to execute ecdysteroid-triggered events and have tracheal defects [137]. HR78 coordinates with the SAM domain co-repressor Moses to help regulate cell proliferation, with HR78 activity favoring and Moses activity inhibiting cell division [138]. The *HR78* pro-proliferation function in the fly is somewhat reminiscent of vertebrate TR2/TR4 function in promoting cell proliferation in the mouse.

The *C. elegans* genome project identified *nhr-41* as the nematode NR2CD ortholog [25]. Like *HR78*, *nhr-41* is broadly expressed and oscillates with the molting cycle. Knockdown of *nhr-41* results in SDS-sensitive dauer larvae, suggesting a defect in the dauer cuticle [25]. A probable null deletion of *nhr-41* is viable, suggesting that *nhr-41* plays a modest or partially-redundant developmental function in *C. elegans*.

The TR2 gene (NR2C1) of vertebrates was the founding member of this gene class, discovered by low-stringency hybridization from a human testis cDNA library [139]. Deletion of TR2 does not result in testicular or spermatogenesis defects—homozygous null mutant mice are viable and fertile [140]. A recent analysis has demonstrated that it regulates early transcription factors that coordinate the development of retinal progenitor cells and knockout mice have severe vision deficits [141]. In contrast, knockout of the TR4 paralog (NR2C2) has dramatic effects on mouse viability, with homozygotes dying either during fetal development or postnatally, with significant compromises in growth and reproductive behavior [142]. Mutant mice also display severe motor deficits associated with aberrant cerebellar development, establishing a major role for TR4 in neuronal development [143]. TR2 and TR4 appear to play partiallyredundant functions in stem cell proliferation. Mice with both genes knocked out die during embryogenesis with increased apoptosis [144]. Expression of Oct-3/4 and Nanog depend on TR2/TR4, suggesting that TR2 and TR4 function in embryonic stem cell self-renewal and differentiation. Because of their potential as targets for anti-cancer therapies, interest in TR2 and TR4 remains high [145].

The NR2C subfamily illustrates some apparent conservation of NR function. In both flies and vertebrates, family members regulate cell proliferation events and play critical roles in development. In both flies and nematodes, they have regulatory patterns and functions associated with the molting cycle. However, the function in regulation of cell proliferation does not seem to have been maintained in *C. elegans*.

# 9. TLL/NHR-67/TLX (NR2E1)

The *tailless (tll)* gene was originally identified by a traditional forward genetic screen as a terminal/gap gene in *Drosophila* [146, 147]. Deficiency in *tll* results in a failure to develop posterior body segments as well as parts of the brain, thus resulting in embryonic lethality. *tailless* has been shown to interact dynamically with a number of other gap genes including *Krüpple, knirps,* and *hunchback* [148]. Loss of function in *tll* decreases neuroblast formation in the protocerebrum and no boundary is formed between the protocerebrum and the deutocerebrum [148, 149]. The *tll* gene also plays a role in eye development, in both the eye-precursor cells and the embryonic optic lobe [148]. These important roles in neural identity and boundary formation appear to be well-conserved in other animals. Loss of *tll* in the adult fly pars intercerebralis leads to a loss of neuropeptide expression and increased aggression, although the specific developmental effect in this part of the brain has not been described [150].

Identified by the *C. elegans* genome sequencing project, *nhr*-67 is involved in a number of developmental processes including neural development, vulval and uterine development, and cell migration. RNAi knockdown of *nhr*-67 demonstrated a function in cuticle shedding, egg-laying and vulval development [25]. *nhr*-67 is a component of a transcription factor circuit in neural differentiation in the ASEL and ASER sensory neurons [151]. During the L2 and L3 stages, *nhr*-67 functions in the differentiation of pre-VU and anchor cells of the ventral uterus [152]. Defective anchor cells fail to invade the underlying vulval tissue and remain in the cell cycle, generating additional cells [153]. Later in larval development, it plays a role in vulval morphogenesis [154] and in the male it regulates the timing of the migration of the linker cell, which is the male equivalent of the anchor cell [155]. Loss of *nhr*-67 results in embryonic and L1 lethality [152]. In the embryo, *nhr*-67 is expressed in the posterior-most daughters of multiple lineages—reminiscent of the function of *tll* in *Drosophila* embryonic termini—and appears to be responsive to Wnt regulation [156].

The vertebrate ortholog Tlx (NR2E1) was first cloned from a chick cDNA library screen [157]. Tlx is expressed in the developing central nervous system—the telencephalon, diencephalon, eye and nasal placode [157, 158]. Unlike its *Drosophila* and *C. elegans* orthologs, mutations in mouse Tlx are not lethal. Mutant mice display varying levels of anatomical and behavioral abnormalities, including patterning defects in the brain, particularly at the pallio-subpallio boundary [159]. Due to the limbic system's development from these early structures, adult knockout mice show significant behavioral changes, most notably high levels of aggression and poor maternal instincts [160]. This may reflect a conserved function, since the fly pars intercerebralis is thought to be analogous to the vertebrate hypothalamus, and the same cofactor,

Atrophin, participates in aggression in both flies and mice [150, 161]. A variety of retinopathies also result from Tlx mutation, indicating that it plays a critical role in the development and maintenance of retinal cells [162]. Tlx plays a role in neural stem cell self-renewal and adult neurogenesis [163], where it functions upstream of a Wnt signaling pathway [164]. In mutant Tlx mice, neural stem cells undergo precocious maturation to neurons and are therefore quickly depleted, resulting in mice with smaller gyrus and forebrain regions [165, 166]. This premature cell cycle exit phenotype appears opposite to the cell cycle phenotype in the *C. elegans* anchor cell, but suggests that Tlx might play a conserved role in regulating cell cycle decisions associated with differentiation.

In summary, functions in neuron development, optic development, and cell cycle control appear to be conserved among NR2E1 gene family members. Interestingly, the namesake phenotype "tailless" in flies appears to reflect an exapted function, since studies in *C. elegans* and vertebrates have failed to demonstrate a similar broad patterning function.

# 10. UNF/FAX-1/PNR (NR2E3)

The *unfulfilled* (*HR51*) gene was named on the basis of the mutant phenotype in which eclosed adults fail to unfold their wings and exhibit reduced fertility [167, 168]. In mushroom bodies, it functions in specifying neuron identities and regulating axon pathfinding [169]. More recently, *unf* has been implicated in circadian rhythms in *Drosophila* by functioning in the identity of small ventral Lateral Neurons as the master pacemaker [170]. In late stage pupae, *unf* accumulates in the Mushroom Body of the brain and in some lateral neurons near the optic lobes. The MB  $\gamma$ -neurons normally undergo pruning and axon regrowth to form adult-specific neural connections via the TOR pathway, but axons fail to regrow following pruning in *unf* mutants [171]. This function depends on *unf/E75* heterodimers and nitric oxide signaling [172].

The *C. elegans* ortholog, *fax-1*, functions in axon pathfinding in the nerve ring and ventral nerve cord [173]. Mutations in *fax-1* cause loss of expression of neuropeptides, neurotransmitter receptors, and other markers of interneuron identity [174, 175]. Similar to the defective axon regrowth phenotype noted in *unf* mutants, *fax-1* mutants display reduced axon regeneration of an interneuron following laser damage [171]. Thus a conserved function in specifying neuronal identity and axonal growth appears to be a feature of this gene.

The photoreceptor-specific nuclear receptor (PNR; NR2E3) was identified by molecular techniques for genes related to Tlx [176]. PNR is an extreme example of a spatially restricted nuclear receptor, being expressed exclusively in the retina and pineal gland. In rod photoreceptors, PNR plays an important role in cell fate by repressing cone-specific genes [177, 178]. Further, deletions in PNR in mice result in retinal degeneration consistent with flecked retinal degeneration in humans [179]. PNR has been shown to interact with Rev-Erb, probably as a heterodimer [172]. Interestingly, PNR has been shown to exhibit circadian regulatory patterns in zebrafish, suggesting an evolutionarily-conserved PNR/Rev-Erb regulatory module associated with neuron differentiation and rhythmic patterns of gene expression [180]. Mutations in human PNR result in inherited retinal degeneration diseases [181].

NR2E3 family members all seem to play roles in neuronal differentiation—and the common function in axonal arborization points to some more specific conservation of function between *Drosophila* and *C. elegans*. The function in photoreceptor development in vertebrates appears to

be another example of exaptation, although in this case perhaps applying a conserved function in neuron differentiation to a new neuron type.

#### 11. DSF (NR2E4) and HR83/NHR-239 (NR2E5)

There exist two additional gene classes in the NR2E family, but both appear to have been lost from vertebrates. *dissatisfaction* was identified as a mutation that affects sex-specific courtship and neural differentiation in both sexes of *Drosophila*, independent of the *doublesex* pathway [182, 183]. Homozygous *dsf* females show resistance to copulation and fail to lay eggs when they do mate [183]. These phenotypes may be explained by the lack of synapses present in the circumferential muscles of the uterus in mutant *dsf* females. Males also display striking courtship behavior phenotypes, including increased male-male courting, delayed copulation, and defects in abdominal curling during the final stage of courtship. These phenotypes may be explained by the irregular innervation of the ventral longitudinal muscles of abdominal segment 5, particularly the presence of a few large, spherical boutons on each fiber. Thus *dsf* plays important roles in nervous system development. Once thought to be an arthropod innovation, *dsf* orthologs are now apparent in the genomes of lophotrochozoans and echinoderms.

Still another NR2E gene family member, *HR83/nhr-239*, has orthologs in some insects (*HR83*), *C. elegans (nhr-239*), lophotrochozoans, an echinoderm, and a hemichordate, but has not been identified in vertebrates [184]. There is considerable divergence in the LBD of the *nhr-239* clade, and some species-specific orthologs have truncated LBDs. In *C. elegans, nhr-239* is expressed at low levels in a few neurons. Mutations in *HR83* are homozygous viable in the fly, but expression and function have not yet been described.

### 12. SVP/UNC-55/COUP-TF (NR2F)

The *NR2F* class of nuclear receptors is well conserved and has been extensively studied. *Drosophila seven-up* (*svp*) functions in a diverse set of cell specification and transcriptional regulation roles. *svp* was identified in an enhancer trap screen [185]. Loss of *svp* results in transformation of R1, R3, R4, and R6 photoreceptors of the compound eye into R7 photoreceptors. Over expression results in a loss of R7 and R8, indicating that it plays a sufficient role in sensory neuron specification [186]. The gene is also required for specification and development of fat body cells [187], cell proliferation in Malpighian tubule development [188], and proper cardioblast diversification by repressing *tinman* in a subset of cells [189]. *svp* also inhibits ecdysone mediated pathways by forming inactive heterodimers with *EcR*, thus inhibiting transcription mediated by *usp/EcR* dimers [190].

The *C. elegans* ortholog, *unc-55*, plays a role in synaptic patterning and male mating circuits [191, 192]. The VD motor neurons of *unc-55* mutants are transformed to resemble the afferent and efferent synaptic patterning of DD motor neurons. Therefore, *unc-55* functions in specifying neuronal identities, similar to the role of its *Drosophila* ortholog.

The vertebrate orthologs, chicken ovalbumin upstream promoter-transcription-factors (COUP-TFI and COUP-TFII; NR2F1, NR2F2), play a significant role in epithelial differentiation in embryogenesis. COUP-TFI was discovered as a transcription factor that regulates

expression of ovalbumin [193, 194]. Human COUP-TFII was identified by its homology to COUP-TFI [195]. The COUP-TFs have overlapping but unique expression patterns. COUP-TFI has high expression in the nervous system and many developing tissues [195]. In particular, COUP-TFI is highly expressed in organs that develop by epithelial proliferation and differentiation, including those that develop by mesenchymal differentiation to epithelium. Mouse COUP-TFI mutants show dose-dependent embryonic lethality or lethality shortly after weaning, reduced growth of the head and heart, severe hemorrhage, edema, enlarged blood vessels, defective angiogenesis, ocular defects, bone defects, and vascular remodeling [194]. COUP-TFI mutants are also missing cortical layer IV in the cerebral cortex lamina, due to a failure of axons to extend to their cortical targets, and resulting excessive cell death [196]—thus a role in neuronal development appears to be evolutionarily-conserved. COUP-TFII mutants have low Angiopietin-1 (Ang-1) levels, which may account for heart and vasculature defects. Defects in the glossopharyngeal nerve result in poor sensation and motor function of the tongue and pharynx, contributing to malnutrition and perinatal death.

The comparison of functions of NR2F family members suggests themes of both exaptation and conservation. A general role in nervous system development is evident among species investigated in detail, but various different roles in organogenesis are apparent in flies and vertebrates.

#### **13. ERR (NR3B)**

The estrogen-related receptor (*ERR*) gene of *Drosophila*, although evolutionarily-related to steroid receptors of great interest to human health, has not been fully characterized. This gene class has been lost in nematodes, but is retained in vertebrates (Figure 1, Table 1). Mutations in *ERR* are lethal in larval development, indicating that the gene plays an essential function. A recent study showed that *ERR* is required for normal spermatogenesis in the fly [197], but the essential function remains undescribed. *ERR* orthologs are present in Lophotrochozoa and Echinodermata, but were apparently lost from nematode lineages.

Vertebrates have three ERR orthologs ( $\alpha/\beta/\gamma$ ; NR3B1, NR3B2, NR3B3), none of which are known to bind a ligand. Knockouts of ERR $\alpha$  in mice are viable and fertile, displaying only modest metabolic defects [198]. The ERR $\beta$  paralog is required for normal placental development and function; gene knockout results in embryonic lethality [199]. Knockouts of the ERR $\gamma$  ortholog present with significant physiological and development defects and result in death in a few weeks [200]. These defects included abnormal heart and spinal cord function [200, 201] and developmental defects in early branching events of the ureteric duct of the kidney [202]. A recent study also described defects in hippocampal neuron physiology and function, but without clear developmental involvement [203]. Further evidence may clarify a role for ERR orthologs in development, but current studies have revealed scant evidence for conserved functions in ontogeny.

#### 14. HR38/NHR-6/NUR77/NURR1/NOR1 (NR4A)

*Drosophila HR38* was identified by molecular cloning as a nuclear receptor with expression associated with morphogenesis [19]. It is broadly expressed, dimerizes with USP/RXR, and

functions downstream of ecdysteroids in a pathway that does not depend on *EcR* [204, 205]. *HR38* does not appear to have a critical role in embryogenesis, but is required for adult cuticle formation during later pupal morphogenesis [206, 207]. Recent evidence has also indicated a regulatory role for *HR38* in oscillatory patterns associated with circadian rhythms [208].

The *C. elegans* NR4A member, *nhr-6*, functions in the development of the spermatheca. In conjunction with the transcription factor Jun, it is required for cell cycle progression in spermathecal cells, thus leading to a defect in cell proliferation and a significant reduction in fertility [209–211]. Null alleles do not display molting defects, suggesting that this class of nuclear receptor has been repurposed to unrelated functions among Ecydsozoa, yet the role (however circumscribed) in cell proliferation does allow some comparison to vertebrate NR4A gene function.

Vertebrates boast three NR4A paralogs: Nur77 (aka NGF1B), Nurr1, and Nor-1 (NR4A1, NR4A2, NR4A3). They were first identified molecularly as immediate-early response genes [212]. Much of the work on this gene class has focused on cell biological studies of their role in metabolism, cell cycle control, and apoptosis [213–215]. Nur77 interacts with RXR and p53 to regulate genes associated the cellular proliferation and participates with RXR and Bcl-2 in regulating apoptosis via a non-transcriptional pathway [214]. While Nur77 has both pro-and anti-proliferative functions, loss of Nurr1 and Nor-1 both lead to net proliferation of cells, suggesting a tumor suppressor role for the latter two. Mice carrying mutations in both genes suffer from rapid-onset acute myeloid leukemia, dying after a few weeks with liver and hematopoietic defects [216]. Thus vertebrate NR4A genes play a major role in regulating hematopoietic stem cells. Although various NR4A knockouts in mice are viable at birth, the Nurr1 gene has been implicated in neuronal differentiation [217]. Other studies have pointed to a role for Nur77 in the differentiation of testicular Leydig cells in mice [218], reminiscent of the defined function of *nhr-6* in the *C. elegans* spermatheca. Therefore, there is some hint of conserved functions between nematodes and mice, but overall the theme for NR4A subfamily members is one of exaptation.

# 15. FTZ-F1/NHR-25/SF-1/LRH-1 (NR5A) and HR39 (NR5B)

The NR5 family consists of one broadly-conserved gene family member (NR5A) and a second gene (HR39/NR5B) that appears to have been lost in nematodes and vertebrates (Table 1). The *ftz-F1* gene of *Drosophila* was first identified on the basis of its biochemical activity as a factor that bound the "zebra element" controlling segmental patterns of *ftz* homeobox gene expression in the embryo [219, 220]. During embryogenesis, maternally-produced Ftz-F1 protein interacts with Ftz protein to regulate downstream gene expression [221–223]. A second isoform functions in larval and pupal development [224]. Here, *ftz-F1* is required for molting and morphogenesis; mutants arrest at larval molts and exhibit defects in adult head eversion, leg elongation, and salivary gland development during the transition to pupa [225, 226]. In the nervous system, Ftz-F1 represses the other NR5 family member, HR39/NR5B, which in turn represses EcR (NR1H) transcription in mushroom body gamma neurons during morphogenesis [227, 228]. In these cells, *ftz-F1* functions in pruning and remodeling of excess neurites.

As in *Drosophila*, the *C. elegans* ortholog *nhr-25* is required for a wide variety of developmental processes, including embryogenesis, vulval and gonadal development, and timing of life cycle events [229–231]. Embryos deficient for *nhr-25* arrest during elongation in midembryogenesis, with defects in hypodermal cell fusion [232, 233]. During larval development, NHR-25 acts in combination with the LIN-39/Dfd homeodomain in vulval precursor cells [233]. While this is reminiscent of the Ftz/Ftz-F1 interaction in *Drosophila*, the coordinating homeobox genes are not orthologs. It also functions in gonad morphogenesis and regulation of germline cell division [229, 232]. *nhr-25* is required for the larva-adult transition and interacts in a complex pathway with heterochronic genes [231], analogous to the morphogenesis requirement for *Drosophila*. Therefore, a role in the coordinated timing of events related to molting and developmental transition appears to be conserved in Ecdysozoa. In other developmental pathways, *nhr-25* may synergize with or antagonize Wnt signaling [230]. This is particularly interesting since *Drosophila* Ftz-F1 regulates various patterning genes including *engrailed*, but has not been directly implicated in *wingless* signaling [234].

Two vertebrate members of NR5A subfamily have been identified. Steroidogenic Factor-1 (SF-1; NR5A1) was first identified by molecular techniques as a transcription factor associated with regulation of steroid hydroxylase genes [235, 236]. It is expressed in gonads, adrenals, and the pituitary [237], where it plays a critical developmental role in the development of steroid-producing and responsive tissues. Loss of SF-1 results in absence of adrenal glands and gonads, indicating a critical role in organogenesis [238]. In the gonads of mice, SF-1 function is required for a range of masculinization events [239]. It plays a similar role in the juvenile-to-adult switch of gonadogenesis in zebrafish [240], reminiscent of its function in early metamorphosis in the fly. In zebrafish, it appears to function upstream of Wnt signaling, similar to *nhr-25*. Specific knockout of SF-1 in the pituitary results in loss of follicle-stimulating hormone and luteinizing hormone-producing cells, with concomitant under-development of gonads [241]. SF-1 is required for differentiation of the VMN ventromedial hypothalamic nucleus cells, where it also mediates the normal circuitry between the hypothalamus and limbic portions of the telencephalon [242]. While not precisely the same, these functions are reminiscent of neuronal functions described in *Drosophila*.

LRH-1 (NR5A2) is expressed in endodermal tissues, including the intestine, liver and pancreas, as well as the ovary and brain [243]. It is essential in mice due to multiple requirements in development of organs, perhaps due to its role as a key regulator of pluripotency in early stem cells [244–246]. It binds to  $\beta$ -catenin and thus is an apparent target of Wnt regulation, like several other NR5 nuclear receptors [247]. In the pancreas, LRH-1 functions in the formation of the multipotent progenitor cell, which eventually gives rise to a number of different pancreatic cell types [248]. In zebrafish, LRH-1 also functions in gut, liver, and pancreas development and synergizes with Wnt signaling pathways [249, 250]. Both SF-1 and LRH-1 bind and respond to phospholipids [251].

Less is known about *Drosophila HR39*, which is absent from *C. elegans* and vertebrates, but present in lophotrochozoans and echinoderms. In addition to the aforementioned role in nervous system development, *HR39* also functions in the development of female reproductive glands [252], once again emphasizing a role in gonadogenesis for this gene class. Because *HR39* mutants are viable, the gene does not appear to play an essential role in life cycle transitions.

In summary, the role for NR5A subfamily members in gonadogenesis appears to be fairly well-conserved. Furthermore, subfamily members have broad functions in organogenesis and life cycle transition. Specific examples of exaptation are also evident, such as the role of Ftz-F1 in segmental patterning in *Drosophila*.

### 16. HR4/NHR-91/GCNF (NR6A)

The *Drosophila HR4* gene was identified as a nuclear receptor that is ecdysone-responsive. Like its vertebrate ortholog GCNF, *HR4* is essential for embryogenesis—although embryos arrest late with cuticular defects [224]. Disruption of *HR4* during larval development results in premature pupariation and arrest in early metamorphosis [61, 224]. It is required for proper upregulation of *FTZ-F1* and operates in conjunction with *HR3* to execute an early temporal switch in the progression from larva to pupae. Some of this function is mediated by the protein's movement from cytoplasm to nucleus in the prothoracic gland, where it inhibits the expression of ecdysone [253]. Thus *HR4* functions both downstream and upstream of the major temporal regulatory hormone in *Drosophila*. Roles for *HR4* in stem cell development or gametogenesis have not been described.

The *nhr-91* gene of *C. elegans* was first identified based on its orthology to GCNF [25]. It is broadly expressed in epithelial cells including the vulva, the spermatheca, excretory cell and a few neurons. The function of *nhr-91* has not been well characterized—attempts to reduce function by RNAi do not cause the severe arrest phenotype observed in mice and flies (although a lethal allele has been reported). *nhr-91* is a target of miR-235 miRNA, where it plays a role in the transition from L1 to L2 larval stages [254]. Expression of *nhr-91* is sharply up-regulated in response to activation of the larval feeding. These observations hint at a possible broad temporal regulatory role like that seen in *Drosophila*.

The Germ Cell Nuclear Factor (GCNF; NR6A1) gene was identified in the early 1990's as a nuclear receptor expressed in mouse sperm [255]. Its function in gametogenesis and embryogenesis has been extensively studied in mice, frogs, and vertebrate tissue culture systems. Mouse knockouts cause homozygous embryonic arrest due to cardiovascular defects, anterior-posterior patterning disruptions, and defects in the development of somites [255]. It also plays a key role in nervous system development—including the normal differentiation and proliferation of neurons in the isthmic organizer and neural stem cells in the early neuroectoderm [256, 257]. In multiple cells, GCNF is a potent transcriptional repressor of the POU homeodomain protein Oct4 [255, 257]. In embryogenesis, expression of Oct4 is normally limited to the germ-line after gastrulation. Loss of GCNF causes ectopic expression of Oct4, thus broadly inhibiting the differentiation of various cells [255]. GCNF also plays multiple roles in both spermatogenesis and oogenesis [258, 259]. A role for GCNF in broad temporal developmental events has not been described. Therefore, the overall theme for the NR6A subfamily is one of exaptation of function over evolutionary time.

# 17. Themes of Conservation and Exaptation in Nuclear Receptor Evolution and Development

Studies of homeodomain and HLH transcription factors have revealed broad conservation of functions in development across animal phyla. For example, the homeobox genes play similar roles in patterning anterior-posterior embryonic development in flies, worms, and mice [13], and can even substitute for each other functionally [260]. Likewise, HLH transcription factors play conserved roles in muscle development and control of cell division [261, 262]. In most cases, mechanistic details differ from one species to another, so patterns of conserved functions may be general or extend to details of cellular pathways [263]. Given the early evolution and strong conservation of nuclear receptors, one might expect to discover similar themes of conserved function in animal development.

Many nuclear receptors play central roles in coordinating broad developmental responses to life cycle transitions such as larval molting and morphogenesis (Table 2; Figure 2). This includes 1D (E75), 1E (E78), 1F (HR3/nhr-23), 1H (EcR), 1J (daf-12), 2B (usp), 2C (HR78), 4A (HR38/nhr-6), 5A (Ftz-F1/nhr-25/SF-1), and 6A (HR4/nhr-91). The nuclear receptors seem particularly well-suited to such developmental events, since they may combine both ligandresponsiveness and transcriptional regulation into a single protein—an arrangement that should be effective for a coordinated whole-animal event. However, it is worth noting that several of these life cycle regulators do not have known ligands (Table 1). Moreover, aspects of these regulatory circuits differ somewhat even among insects [224]. In vertebrates, where life cycle transitions are less dramatic and molting is not required, these nuclear receptors regulate a range of different functions in cell differentiation and organogenesis. Therefore, the lack of sharp, dramatic life cycle transitions, such as molting and morphogenesis, can be seen as an opportunity for vertebrate repurposing of nuclear receptors to new functions. At the same time, some of these same nuclear receptors play roles in embryogenesis, nervous system development, and control of biological rhythms that are more broadly conserved (e.g. E75 and Rev-Erb in rhythms), suggesting that these may represent ancestral functions.

Class	D. melanogaster	C. elegans	M. musculus	
1D	E75	NHR-85	Rev-Erb α/β	
	molting, rhythms, metamorphosis, oogenesis	molting, life cycle, female organogenesis	rhythms	
1E	<i>E</i> 78	SEX-1	(ortholog absent)	
	female organogenesis, oogenesis, gastrulation, metamorphosis	female sex determination, female organogenesis		
1F	HR3	NHR-23	ROR α/β/γ	
	embryogenesis, metamorphosis, nervous system, trachea, cuticle, wings	molting, life cycle, cuticle	nervous system, immune system, rhythms, eyes	
1H	EcR	(ortholog absent)	LXR $\alpha/\beta$	
	embryogenesis, metamorphosis, gut, male nervous system		nervous system, immune system	

Table 2: Functions of Conserved Nuclear Receptors in Development.

1J	HR96	DAF-12/NHR-8/-48	VDR/PXR/CAR	
	(none known)	life cycle, longevity	female organogenesis, immune system, bones	
2A	HNF4	superfamily	ΗΝF4 α/γ	
	(none known)	excretory tube, germ-line, embryogenesis, hypodermis, muscle	gastrulation, liver, nervous system	
2B	USP	(ortholog absent)	RXR α/β/γ	
	embryogenesis, metamorphosis, gut, male nervous system		embryogenesis, heart, male spermatogenesis, neurogenesis, eyes, rhythms	
2C	HR78	NHR-41	TR2/TR4	
	molting, trachea, larval growth	cuticle	eyes, fetal growth, nervous system, embryogenesis, stem cells	
2E	TLL	NHR-67	TLX	
	embryonic patterning, nervous system, eyes	embryogenesis, nervous system, female organogenesis, cell migration, cell division	nervous system, stem cells, eyes	
2E	DSF	(ortholog absent)	(ortholog absent)	
	nervous system			
2E	UNF	FAX-1	PNR	
	nervous system, rhythms	nervous system	eyes	
2E	HR83	NHR-239	(ortholog absent)	
	(none known)	(none known)		
2F	SVP	UNC-55	COUP-TF α/β/EAR2	
	eyes, fat cells, heart	nervous system	embryogenesis, heart, vascular system, nervous system, bone, eyes	
3B	ERR	(ortholog absent)	ERR α/β/γ	
	spermatogenesis		embryogenesis, heart, nervous system, kidney	
4A	HR38	NHR-6	NUR77/NURR1/NOR1	
	cuticle, morphogenesis, rhythms	male organogenesis, cell division	cell division, apoptosis, hematopoiesis, nervous system, liver, male organogenesis	
5A	FTZ-F1	NHR-25/-97/-119	<i>SF-1/LRH-</i> 1	
	embryonic patterning, molting, morphogenesis, nervous system	embryogenesis, female organogenesis, hypodermis, life cycle	gonads, life cycle, adrenals, pituitary, nervous system, stem cells, gut, liver, pancreas	
5B	HR39	(ortholog absent)	(ortholog absent)	
	female organogenesis			
6A	HR4	NHR-91	GCNF	
	embryogenesis, cuticle, metamorphosis	life cycle	embryogenesis, gametogenesis, nervous system, heart	





Selected key organ systems and broad patterning functions for nuclear receptors in development are shown. Color scheme at right identifies specific NRs by overall class. Some functions are indicated at the temporal stage at which they are observed, but may function earlier. Genes assigned to the "pattern" class may have roles in specifying embryonic region (*e.g. tll*), major embryonic events such as gastrulation (*e.g. E78*), or early cell divisions (*e.g. LRH-1, TR2*). Illustrations are not to scale. Artwork is by S. Bodofsky.

Some of the NRs with the strongest conservation and representation in different animal phyla are those of the NR2 group. This includes nuclear receptors such as *tailless* and COUP-TF that have extensively-studied functions in regulating development. The suggestion that a conserved regulatory pathway involving fly *tll* and vertebrate *Tlx* in the development of neuronal systems for aggressive behavior is particularly intriguing [161]. Evidence from worms, flies, and mice also suggests that *nhr*-67 and Tlx play similar roles in regulating cell division and neuron development [148]. The NR2E3 family (*unf/fax-1*) plays a conserved role in neuronal specification and regulation of neurite arborization. However, even with these more conserved

NRs there are still clear examples of exaptation. For example, the functions of *tll* in embryonic patterning in *Drosophila* and *nhr*-67 in uterine development in *C. elegans* do not seem to be functions for which there are exact correlates in other phyla. Overall, the NR2 receptors are heavily utilized for developmental functions, particularly in embryogenesis and nervous system development (Figure 2).

Eye development is a major theme in vertebrate nuclear receptor developmental function. ROR $\beta$ , RXR $\alpha$ , TR2, TLX, PNR and COUP-TF $\alpha$  all play critical roles in the patterning, differentiation, or survival of retinal cells (Table 2; Figure 2). This may reflect a specialized role for retinoid signaling in the developing vertebrate eye, however *tll* and *svp* both function in *Drosophila* eye development, which is not a homologous process and is not dependent on retinoids. Nematodes do not have eyes and it is not clear what cells have an equivalent ontogenetic origin. Nonetheless, the common function of *tll*/Tlx and *svp*/COUP-TF $\alpha$  suggests an evolutionarily ancient function in eye development.

The NR1 group appears to be more plastic in terms of sequence and, in at least one case, function. The NR1D-E-F and H-I-J groups are difficult to organize into well-supported clades due to lower overall sequence conservation [Figure 1]. Our evaluation of representative members of each orthologous gene represents a parsimonious interpretation of their relationships. However, common regulatory relationships and functions among *EcR* in *Drosophila* (NR1H), *daf-12* in *C. elegans* (NR1J), and estrogen receptor in vertebrates (NR3A) [89, 90] suggest that functional pathways mediated by NR1 genes can be swapped to other NRs over evolutionary time. That is, individual NRs can repurpose to take on functions performed by another NR. Therefore, in some cases conserved functions may not be accomplished by strict orthologs in different species.

Conservation at the level of sequence is greatest in the DBD, consistent with observations that protein-DNA binding relationships have been maintained over large evolutionary distances [264, 265]. By contrast, the LBD is considerably less conserved, especially in *C. elegans* as compared to other animals [106, 184]. This provides some flexibility in terms of potential ligand control as well as co-activators and co-repressors. Furthermore, exaptation may be accomplished at the level of regulation of the NR itself or in variation of the downstream targets that possess a conserved DNA binding site for a given NR. Knowledge of the precise targets of NR regulation in multiple species should help provide an understanding of these modes of evolutionary change.

Overall, the theme of nuclear receptor function in development emphasizes exaptation over functional conservation. A few examples, such as the *Drosophila E75* and vertebrate Rev-Erb conserved function in circadian rhythms and possible conserved functions in neuronal developmental pathways between *Drosophila tll* and vertebrate *Tlx*, indicate that some functions have been maintained over a long history of animal evolution. But there are many examples of exaptation. In particular, the vertebrate immune system has recruited several nuclear receptors, including ROR, LXR and VDR, for regulating key steps in cellular differentiation or survival. Functional relationships among conserved nuclear receptors will benefit from studies that focus on functions in additional taxa such as Lophotrochozoa.

### **Competing Interests**

The authors declare no competing interests.

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