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Review

Nuclear hormone receptors in nematodes: Evolution and function

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ABSTRACT

Nuclear hormone receptors (NHRs) are proteins that regulate gene expression in response to developmental, environmental, and nutritional signals. The activity of some NHRs is selectively and reversibly modulated by small molecular weight compounds. However, for others – termed "orphan" receptors – no such ligands have (yet) been identified, and at least some NHRs may lack natural ligands. NHRs exhibit a stereotyped architecture, with conserved N-terminal DNA-binding domains (DBDs) and more variable C-terminal ligand-binding domains (LBDs). NHRs control the transcription of remarkably diverse and specific gene networks, apparently by integrating multiple regulatory inputs that interact with distinct receptor surfaces; these inputs include small molecule ligands, transcriptional coregulators, and response elements, the genomic sites to which the receptors bind. NHRs comprise an ancient superfamily found in all metazoans, and recent findings have revealed NHR-like regulatory factors in fungi. Here, we consider NHR function and evolution in nematodes, roundworms that inhabit terrestrial, marine, and freshwater habitats; we focus in particular on the well-established experimental organism *Caenorhabditis elegans*. Interestingly, the *C. elegans* genome encodes a massively expanded NHR family; we speculate that some of the multiple physiological activities governed by individual mammalian NHRs may be distributed among multiple members of the *C. elegans* family, potentially focusing and simplifying functional analyses. Accordingly, investigations of relevant NHR cofactors, ligands, and response elements might also prove to be simpler; moreover, the abbreviated intergenic regions of the *C. elegans* genome will facilitate the assignment of response elements to target genes. Finally, the growing interest in medically relevant nematodes is providing novel insights into the function and evolution of NHRs.

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Contents

1. The *C. elegans* NHR family – redundancy or specificity? 50
2. NHRs in other nematodes 50
3. Response elements and primary response genes of *C. elegans* NHRs 51
4. Specification of NHR output by coregulators 52
5. Ligands for *C. elegans* NHRs: a party of one 52
6. Conclusions and outlook 53
Note added in proof 53
Acknowledgements 53
References 54

Nuclear hormone receptors (NHRs) are DNA-binding regulatory factors that fine-tune transcriptional output in response to multiple regulatory inputs. The latter include protein–protein interactions (e.g. with transcriptional coregulators), reversible binding to small molecular weight ligands, and sequence specific genomic response elements (Meijsing et al., 2009; O'Malley et al., 2008; Wolf et al., 2008). Accordingly, NHRs affect a plethora of genes in a distinctive and context-dependent fashion. Dissecting such regulatory complexity remains a daunting task of broad biological and medical importance. Over the last decade, researchers have begun to harness genetic model organisms, such as the nematode *Caenorhabditis elegans*, to dissect how these regulatory factors can have such diverse effects depending on the tissue, cell, or gene. Such an approach at first glance may appear curious, because (i) we know extremely little about *C. elegans* NHR ligands, DNA response elements, coregulators, and interacting signal transduction pathways, and (ii) the *C. elegans* genome encodes a NHR family approximately six times larger than the human family, which would presumably

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complicate analysis of this gene family. However, the large NHR family size may in fact indicate that individual *C. elegans* NHRs perform highly specialized functions that have been integrated into a smaller number of more broadly acting NHRs in higher metazoans, although some *C. elegans* NHRs also affect physiology and development broadly. Furthermore, with traits such as a highly compact genome, an invariant, fully mapped cell lineage, well-characterized developmental and physiological processes, powerful genetics with short generation time, facile RNAi and imaging accessibility, and outstanding experimental tractability, *C. elegans* provides an exciting opportunity to study complex regulatory networks in live metazoans. Study of how ligand-binding, coregulators, and response elements have co-evolved with NHR families in individual nematode lineages promises to reveal new insights into NHR biology. Below, we review what is known about NHR signaling in *C. elegans* and other nematodes.

1. The *C. elegans* NHR family – redundancy or specificity?

When the genome sequence of *C. elegans* was completed (*C. elegans* Sequencing Consortium, 1998), one of its more surprising features was an unexpectedly large family of NHRs encompassing 284 genes (Sluder et al., 1999). Despite some early indications to the contrary, it appears that almost all these genes produce proteins and are not pseudogenes (Miyabayashi et al., 1999). The *C. elegans* NHR family is therefore far larger than its human (48 genes), mouse (49 genes), and *Drosophila melanogaster* (18 genes) counterparts (Maglich et al., 2001). The massive expansion of NHRs in *C. elegans* provides an opportunity to study evolution and diversification of this family, but it has been difficult to exploit, as just a handful of NHRs have known functions. Currently, mutation or depletion by RNA interference (RNAi) of 50 *C. elegans* NHRs are known to evoke detectable phenotypes (Harris et al., 2010), but only a few of these have been studied in any real depth.

Despite the sizeable NHR complement, not all six metazoan NHR subfamilies exist in *C. elegans*, as its genome encodes 15 “conserved” NHRs that belong to five of the six NHR subfamilies (NR1, NR2, NR4, NR5, and NR6; reviewed in Antebi, 2006; Sluder et al., 1999; Van Gilst et al., 2002). However, to date, bioinformatics approaches have failed to identify NR3 type (steroid receptor) NHRs in *C. elegans* (Sluder et al., 1999; Sluder and Maina, 2001), and only a single NR3 member has been described in *Drosophila melanogaster*. Thus, perhaps an NR3 gene present before the arthropod-nematode split was lost in the lineage leading to nematodes (Maglich et al., 2001). However, as discussed below, sequence-based homology searches are limited, and have been shown to miss such evolutionary links.

The remaining 269 “divergent” *C. elegans* NHRs appear to have derived from an ancient Hepatocyte Nuclear Factor 4 (HNF4)-related ancestor and have been dubbed Supplementary NHRs (*supnrs*) (Robinson-Rechavi et al., 2005). The *supnrs* have undergone extensive diversification, and most remain uncharacterized.

Interestingly, although representing one of the evolutionarily most ancient NHR types, NR2B type NHRs, have not been detected in the *C. elegans* genome (Maglich et al., 2001). The mammalian NR2B member RXR serves as heterodimer partner for numerous NHRs (Mangelsdorf and Evans, 1995). It is possible that a *C. elegans* RXR functional homolog exists but has not been recognized. Alternatively, the HNF4-derived *supnrs* and some of the conserved *C. elegans* NHRs may mimic the propensity of HNF4 to exclusively form homodimers. Predominant *C. elegans* NHR homodimerization is supported by a comparative study of LBD residues that specify dimerization (Brelivet et al., 2004), although we note that the DBD may also contribute to dimer formation (Kumar and Chambon, 1988). Brelivet et al. found that the LBDs of most *C. elegans* NHRs contain amino acids that mediate homodimerization and lack the residues that typically enable heterodimerization. However, emerging experimental evidence suggests that at least some *C. elegans* NHRs can form heterodimers (Li et al., 2004, and ST & KRY unpublished). Whether any of these dimers is relevant *in vivo* remains to be determined. If heterodimerization were common amongst *C. elegans* NHRs, it would greatly expand the number of combinatorial regulatory outputs that could be achievable by this large gene family.

The NHR family expansion begs the question why the genome of a soil nematode encodes such a large complement of these regulatory factors. Interestingly, the gene duplication rate in the *C. elegans* genome is ~10-fold higher than that in *Drosophila*; 32% of *C. elegans* genes have at least two paralogs (Gu et al., 2002; Lynch and Conery, 2000). Thus, it seems likely that tandem gene duplication drives genetic diversity in *C. elegans.* While there is no chromosome or arm bias to the phylogenetically conserved *C. elegans* NHRs, the *supnrs* are distinctly non-random, with 149 residing on chromosome V, and strong clustering on the recombinogenic autosome arms (Thomas, 2006); the duplications have occurred over a long evolutionary timescale (Thomas, 2006). In principle, the duplicated genes could then undergo (i) nonfunctionalization, whereby mutations in one copy render it inactive; (ii) neofunctionalization, whereby one copy acquires a novel function, while the other copy maintains its original functions; or (iii) subfunctionalization, whereby mutations in both copies reduce their cumulative function to the level of the ancestral gene (Lynch and Conery, 2000; Lynch, 2002). In the case of the *C. elegans supnrs*, the latter two fates seem more likely, as the duplicated genes have been maintained and most appear to be expressed. Given that genes involved in xenobiotic responses (e.g. glutathione-S-transferases and cytochrome P450s (Thomas, 2008)) and innate immunity (e.g. lectins (Thomas, 2008)) are also massively duplicated in *C. elegans*, one speculation is that certain *supnrs* may refine the sensing and response to xenobiotic compounds, while others may have undergone subfunctionalization. In this view, even NHRs with closely related sequences may affect distinct gene sets, or perhaps act in different cell types to perform rather tightly focused roles.

2. NHRs in other nematodes

The size of the NHR family in *C. elegans* raises the question whether other nematodes encode similarly expanded NHR families. Although an accurate answer awaits the sequencing of more nematode genomes, the genomes of *C. briggsae* and *C. remanei* encode 232 and 256 putative NHR genes, respectively (Haerty et al., 2008). This suggests that, at least in the *Caenorhabditis* genus, the NHR family expansion may be shared feature. Thus, concepts and principles discovered for the *C. elegans* NHRs may apply to related species as well.

The genomes of *C. elegans*, *C. briggsae*, and *C. remanei* are surprisingly divergent, given that they belong to the same genus, with only approximately 10,000 genes showing strict orthology (Thomas, 2008). Although significant numbers of NHRs are conserved between *C. elegans*, *C. briggsae*, and *C. remanei*, many appear to have diverged: Haerty et al. found that approximately half of the NHRs in each *Caenorhabditis* are conserved as three-way reciprocal orthologs, with another 10% exhibiting two-way relationships with one other species (Haerty et al., 2008). They also noted that NHRs appear to have evolved by rapid tandem duplications, followed by lineage specific loss or expansion, and suggested that the non-orthologous NHRs may have arisen from lineage-specific gene duplications. It is intriguing to consider that NHRs contribute to speciation, thus allowing each nematode species to thrive in a certain environmental niche. However, given that many *C. elegans*

Table 1
NHR families in nematodes.

Species	NHR genes	Total gene number (best estimates)	Life style	Reference
C. elegans	283	20,186	Free-living microbivore	*C. elegans* Sequencing Consortium (1998)
C. briggsae	232	19,507	Free-living microbivore	Haerty et al. (2008) and Stein et al. (2003)
C. remanei	256	~20,000	Free-living microbivore	Haerty et al. (2008)
M. incognita	92	19,212	Root knot nematode	Abad et al. (2008)
M. hapla	76	14,420	Root knot nematode	Opperman et al. (2008)
B. malayi	27	~14,500–17,800	Filarial human parasite	Ghedin et al. (2007)

nhr genes reside on the chromosomal arms – which are enriched for rapidly evolving (duplicated) genes (Thomas, 2008) – we cannot rule out the hypothesis that such duplications may result from structural and not adaptational causes.

Outside of *Caenorhabditis*, relatively little is known about nematode NHRs. Although several nematode genomes are currently being sequenced, few high quality datasets are available for evolutionary comparison. Nevertheless, the completed genomes of the plant parasites *Meloidogyne hapla*, *M. incognita*, and the human parasite *Brugia malayi* provide some insights (Abad et al., 2008; Ghedin et al., 2007; Opperman et al., 2008). These nematodes encode a similar number of genes as the *Caenorhabditid* species (estimated ~15,000–20,000 genes; Table 1). Yet, their NHR families are significantly smaller (~30–90 genes; Table 1), albeit still larger than the NHR family encoded by *D. melanogaster* (18 genes). Interestingly, the *B. malayi* genome encodes RXR/USP, a conserved NHR of the NR2B subfamily, which is absent in *C. elegans*, indicating that this NHR has been lost following the *C. elegans*–*B. malayi* split. However, like *C. elegans*, neither *B. malayi* nor *M. incognita* appear to encode NR3 type NHRs (Abad et al., 2008; Ghedin et al., 2007). Furthermore, *B. malayi*, *C. elegans*, and *M. incognita* share a group of *supnrs*, with *M. incognita* exhibiting a species-specific expansion of one *supnr* subgroup (Abad et al., 2008). Based on these findings, Abad et al. suggested that *supnr* expansion started before the evolutionary *Caenorhabditid*–*Meloidogyne*–*Brugia* separation and continued thereafter. Pending experimental verification of predicted similarities, it appears that some conserved NHRs may also predate this split, with subsequent loss in the *Caenorhabditid* species.

Several explanations have been proposed for the differences in NHR gene count between nematode species. Such variations may reflect the niche specialization of internal plant (*Meloidogyne* spp.) or vertebrate (*B. malayi*) parasites, as dwelling within another organism provides a well-defined, homeostatic environment compared to a soil-dwelling life-style (Abad et al., 2008; Ghedin et al., 2007; Opperman et al., 2008). Alternatively, it has been suggested that internal parasites may be under pressure to reduce genome content, whereas *Caenorhabditid* species may have to deal with a multi-faceted and ever-changing habitat.

In human physiology, NHRs are attractive targets for therapeutic intervention via small molecule compounds. Currently, a significant amount of research is dedicated to the development of high-affinity/high-specificity ligands that selectively promote or inhibit NHR action. The expansion of the NHR family in nematodes may present an opportunity for targeted therapeutic intervention, especially to treat parasitic nematode infestations. The fact that NHRs appear to be evolving rapidly, with significantly diverged NHR families in closely related species, suggests that it may be possible to develop highly selective compounds to modulate the activity of NHRs critical to parasite viability or pathogenicity.

Infectious larvae in parasitic nematodes are an alternative life stage highly reminiscent of the dauer stage in non-parasitic soil dwelling nematodes. In the latter species, the dauer stage represents a specialized larval stage that emerges in response to food deprivation or overcrowding. Dauer larvae are adapted for survival and dispersal; accordingly, dauer formation is regulated by an intricate signaling network wherein the NHR DAF-12 plays a key role. Intriguingly, two recent studies showed that the formation of infectious larvae in two *Strongyloides* species uses a similar signaling network converging on a DAF-12 like NHR (Ogawa et al., 2009; Wang et al., 2009). Accordingly, treatment with the DAF-12 ligand Δ7-dafachronic acid (DA) but not the structurally related ligand Δ4-DA blocked formation of infectious larvae. Wang et al. further demonstrated that DAF-12 orthologs from other parasitic nematode species also bound Δ7-DA. Both groups of authors suggested that DAF-12 related NHRs may represent targets for pharmacological intervention, noting the deep evolutionary origin of the DA-DAF-12 ligand:receptor pair, and the obligatory requirement for many parasitic nematodes to pass through a dauer-related stage (Ogawa et al., 2009; Wang et al., 2009). Although this requirement will need to be verified for individual parasitic nematodes, these reports suggest an exciting strategy to combat a global health problem.

3. Response elements and primary response genes of *C. elegans* NHRs

Although the functions of some *C. elegans* NHRs are emerging, the genomic binding sites, response elements, and regulatory targets of almost all nematode NHRs remain unknown. However, below we point out the studies wherein investigators identified targets and/or binding sites for NHRs. We also note that several gene families known to represent physiological targets for mammalian NHRs are expanded in *C. elegans*, like the *nhr* gene family itself; these include C-type lectins, cytochrome P450 (*cyp*) genes, ABC-transporters, glutathione-S-transferases, UDP-glycosyl/glucuronosyl-transferases, ion channels, and acyl-transferases (Thomas, 2008). Whether regulatory relationships exist between *C. elegans* NHRs and these genes remains to be determined, but it is intriguing to ponder certain connections. For example, some *cyp* gene products participate in the synthesis of high-affinity NHR ligands, which makes it tempting to propose feedback/feedforward signaling between NHRs and *cyp* genes. Also, several of the above gene classes encode proteins that metabolize and eliminate foreign contaminants (Xu et al., 2005). As at least one *C. elegans* NHR can contribute to xenobiotic responses, it has been suggested that NHRs could have multiplied and diverged in part to deal with the diversity of foreign molecules a free-living soil organism might encounter (Lindblom et al., 2001; Lindblom and Dodd, 2006).

Bona fide binding sites have been identified for only one *C. elegans* NHR, DAF-12, by *in vitro* genomic selection following validation with fluorescence anisotropy (Shostak et al., 2004). Putative binding sites were identified for NHR-31, a *supnr* involved in patterning the nematode excretory system, by bioinformatic analysis of the promoters of NHR-31 regulated genes uncovered via microarray analysis (Hahn-Windgassen and Van Gilst, 2009); these sites have not yet been functionally validated. We note that the compact nature of the *C. elegans* genome, with extremely short intergenic sequences and a very high gene density, should simplify such validation studies; indeed, these features provide yet another advantage of this model organism relative to other metazoan models.

Remarkably, there is to date no report of chromatin-immunoprecipitation (ChIP) of a *C. elegans* NHR, despite the fact that recent technological breakthroughs (ChIP coupled to either microarray [ChIP-Chip] or massively parallel sequencing [ChIP-Seq]) have greatly advanced the sensitivity and coverage of this method and led to important insights into the genome-wide action of regulatory factors (Park, 2009). The model organism ENCODE (modENCODE) project provides a unified platform for genomic studies that may yet motivate investigations focusing on nematode NHRs (Celniker et al., 2009). From an evolutionary standpoint, it would be especially interesting to define the genomic binding sites, response elements, and the target genes directly regulated by some of the highly divergent NHRs. Van Gilst et al. reported that many *C. elegans* NHRs contain P-boxes (DNA recognition motifs within the DBD) that are unique to this organism (Van Gilst et al., 2002). Unveiling their genomic targets could provide insight into NHR evolution and into the overlap and separation of gene regulation by closely related NHRs.

4. Specification of NHR output by coregulators

Transcriptional regulation depends on multiple players; these include response elements, genomic loci that interact with sequence specific regulatory factors such as NHRs. In turn, these regulatory factors recruit transcriptional coregulators, specifying the combinatorial assembly of regulatory complexes that differ as a function of gene and cell contexts, and that integrate the effects of multiple signaling inputs. Thus, one way to gain insight into the action of individual *C. elegans* NHRs is to study their physical and functional interactions with coregulators; a logical extension of this approach would also define distinct interaction surfaces within conserved and divergent NHRs. Clearly, much has been learned about mammalian steroid receptor action by studying their coregulators, such as the p160 family (e.g. O'Malley et al., 2008; Rogatsky et al., 2003; Stallcup et al., 2003).

Sequence alignment-based queries have failed to reveal homologs of several prominent mammalian coregulators in *C. elegans* (e.g. p160 family, PGC-1 family, RIP140), whereas others, such as the Mediator subunit MED1, exhibit little sequence conservation with their putative mammalian orthologs (Bourbon, 2008). Curiously, the mammalian homologs of these absent/divergent coregulators are not exclusively linked to the NR3 family that so far has not been detected in the *C. elegans* genome; for example, p160s and PGC-1 are known to also coregulate HNF4α (Rhee et al., 2003; Wang et al., 1998). Thus, it appears that *C. elegans supnrs* utilize different, yet unknown coregulators (see also below). However, as with NR3 type receptors, we point to the limitation of sequence alignment based homology searches, which may fail to recognize relationships between highly divergent proteins. Two examples are illustrative in this context: Although most or all Mediator subunits are broadly conserved in eukaryotes, this was not recognized until iterative PSI-BLAST searches had been completed (Bourbon, 2008). Even more compelling is the case of NHR-like proteins in fungi. Phelps et al. used a protein structure prediction based algorithm to identify yeast proteins that share structural, architectural, and functional aspects of classical NHRs (Phelps et al., 2006). Using such algorithms to search for unrecognized *C. elegans* coregulators might provide new bounty, although their reliance on known functional domains as input may be limiting. In any case, no expansion of a coregulator family akin to the amplification of NHRs has been recognized in *C. elegans* to date. Thus, the vast pool of NHRs may share a comparably small complement of coregulators, and competition for critical coregulators may arise between individual NHRs. Preferential NHR:coregulator interactions could be driven by differential NHR expression in certain cell types, and/or by differential affinities of functional NHR surfaces for a particular coregulator.

Only two coregulators have been identified for *C. elegans* NHRs: MDT-15 and DIN-1S. We showed that NHR-49 interacts with the Mediator complex subunit MDT-15 to regulate the expression of lipid metabolism genes (Taubert et al., 2006). We also found that, although MDT-15 coregulates several sets of NHR-49 targets (fasting response genes, fatty acid desaturase genes), it regulates additional aspects of metabolic biology independent of NHR-49 (Taubert et al., 2006, 2008); similarly, NHR-49 is likely to control some genes in MDT-15 independent fashion. Thus, a particular NHR:coregulator pair selectively influences overlapping but distinct sectors of metabolism. This implies that coregulators, far from being generic activation or repression modules, play precise roles in the combinatorial assembly and function of regulatory complexes that produce patterns of gene expression that are highly specific to a given physiologic context. Consistent with this concept, MDT-15 can also bind NHR-64 and several other NHRs (Taubert et al., 2006; Arda et al., 2010), suggesting that it may integrate signaling by multiple *C. elegans* NHRs, although the functional consequences of these interactions remain to be defined. In all these cases, NHR:coregulator association occurs through the KIX-domain of MDT-15 (Taubert et al., 2006; Arda et al., 2010). KIX-domains are known to bind regulatory factors, but they had not been recognized as interaction targets for NHRs; accordingly, establishing the determinants of KIX:NHR binding may provide insight into the evolution of an NHR:coregulator interaction surface. Recently, the KIX-domain in the yeast homolog of MDT-15, Gal11, was identified as a physical and functional target of NHR-like transcription factors in yeast (Thakur et al., 2008, 2009), implying that the NHR:MDT-15/Gal11 interaction is ancient and evolutionarily conserved. It is alternatively possible that yeast NR-like factors and *C. elegans* NHRs may share related cofactors due to convergent evolution. In all these cases, it will be important to identify the interaction surfaces using genetics and structural approaches; this will define the molecular and structural determinants that enable physical association between NHRs and their coregulators.

The other recognized *C. elegans* NHR coregulator is DIN-1S, which binds the NHR DAF-12 (Ludewig et al., 2004). Importantly, this association is ligand-dependent as the cholesterol-derived DA ligands mentioned earlier cause dissociation of DIN-1S from DAF-12 (Motola et al., 2006). Interestingly, DIN-1S contains variants of the known LxxLL interaction motif (L/IxxI/V/L), as well as LxxLL motifs; however, it is not known whether these motifs are relevant for DAF-12:DIN-1S interaction. Nevertheless, these reports confirm that ligands modulate NHR:coregulator interactions, and strongly hint at hormone-regulated gene expression in *C. elegans*.

Systems biology may aid the discovery of additional coregulators for *C. elegans* NHRs. Using large-scale yeast-two-hybrid approaches, Li et al. identified several proteins that interact with two or more NHRs (Li et al., 2004). The identities of some of these suggest that they represent NHR coregulators (e.g. a helicase, an HMG domain protein, a general transcription factor). Other putative coregulators can be inferred by sequence homology, such as p300/CBP, Swi/Snf-related proteins, histone acetyl transferases, and others. Clearly, to understand the evolution of NHR regulatory networks, and the evolution of interaction surfaces in NHRs, it must be a primary goal to identify and characterize novel NHR:coregulator interactions in *C. elegans*.

5. Ligands for *C. elegans* NHRs: a party of one

Virtually all *C. elegans* NHRs contain recognizable LBDs, but currently only one NHR ligand family is known in this nematode, the DAs that bind DAF-12. Genetic data had long supported the idea that

DAF-12 was a ligand-gated regulator (Antebi et al., 2000; Gerisch et al., 2001; Matyash et al., 2001, 2004). For example, loss-of-function mutations in *daf-12* prevent animals from entering dauer. In turn, *daf-12* interacts genetically with *daf-9*, a member of the cytochrome P450 (CYP450) family, which is critical to mammalian steroidogenesis. Finally, cholesterol depletion and cholesterol trafficking defects evoke constitutive dauer larva formation (Gerisch et al., 2001; Jia et al., 2002; Matyash et al., 2004). Thus, it appears that cholesterol derivatives produced in the presence of food prevent DAF-12-dependent dauer entry. Elegant work by the Antebi and Mangelsdorf laboratories demonstrated that DAF-9 and other enzymes metabolize 3-keto-sterols into high-affinity DAF-12 ligands, the DAs (Motola et al., 2006). Indeed, DAF-12 binds two related DAs with similar affinity, Δ7-DA and Δ4-DA. As noted above, these ligands selectively affect larval development in the related nematodes *Pristionchus pacificus* and *Strongyloides papillosus*, suggesting that they differentially regulate gene expression (Ogawa et al., 2009).

Aside from DAs, nothing is known about ligands of the other 283 *C. elegans* NHRs, and whether they do in fact possess ligands remains to be determined. Among the vertebrate NHRs, liganded receptors are scattered throughout the NR1, NR2, NR3, and NR5 (Krylova et al., 2005) subfamilies, but these subfamilies also contain orphan NHRs. Although orphan NHRs may yet turn out to bind ligands, Laudet argued that the orphan state is plesiomorphic, being the presumed state of the ancestral NHR-like molecule and present in all of its derived subfamilies, with ligand binding evolving independently in some of the subfamilies (Laudet, 1997). As more ligands are being discovered for orphan receptors, it may become apparent that the liganded state is ancestral, with the orphan state being derived (Thornton et al., 2003). Based on evidence that the single *Drosophila* HNF4 homolog is liganded (Palanker et al., 2009), we can consider three formal possibilities: (i) ligand binding was acquired by an HNF4 ancestor that emerged after the arthropod/nematode split in the lineage leading to arthropods; or (ii) ligand binding was acquired in an arthropod/nematode ancestor, but later lost in the lineage leading to nematodes; or (iii) ligand binding is ancestral for metazoans, but some HNF4-like receptors lost ligand binding during the expansion of the subfamilies. Reconciling these possibilities will require testing the ligand binding ability of “increasingly ancient” HNF4 ancestors – similar to that performed for the glucocorticoid receptor (GR) ancestor (Ortlund et al., 2007) – and aggressive attempts to test other *C. elegans* receptors for ligands.

It seems likely that additional, perhaps many, nematode NHRs besides DAF-12 are ligand-gated. Molting in *C. elegans* is dependent on both cholesterol and an NHR-23/NHR-25 circuit analogous to the *Drosophila* DHR3-ecdysone receptor circuit (Hayes and Ruvkun, 2006). Given that *Drosophila* molts are controlled by the steroid ecdysone and its cognate NHR (Carney et al., 1997; Koelle et al., 1992), a cholesterol-derived steroidal ligand probably controls molting in *C. elegans*. Although many downstream effectors of NHR-23/NHR-25 have been identified through forward genetic screens, putative ligand synthetic genes upstream of these two NHRs have remained elusive. Interestingly, recent work in the parasitic nematode *B. malayi* by Tzertzinis et al. (2010) describes an NHR pair that functionally resembles the insect USP/RXR. This NHR dimer (Bm EcR/Bm RXR) may contribute to molting in *B. malayi*, thus strengthening the notion that molting in nematodes is a steroid hormone-regulated process (Tzertzinis et al., 2010).

In addition to NHR-23/NHR-25, the mutant and RNAi phenotypes of other *C. elegans* NHRs also hint at the existence of ligands. For example, NHR-8 has been implicated in xenobiotic detoxification and postulated to bind toxins in a fashion reminiscent of the mammalian detoxification NHRs (Lindblom et al., 2001). Also, NHR-49, NHR-80, and potentially several other NHRs are involved in fat metabolism in *C. elegans* (Ashrafi et al., 2003; Brock et al., 2006; Van Gilst et al., 2005a,b). In mammals, NHRs such as HNF4, LRH1, LXR, PPAR, and FXR bind metabolic intermediates, suggesting that metabolic NHRs in *C. elegans* may be similarly liganded. Furthermore, study of the resurrected 450 million year old ancestral molecule of the human GR and mineralocorticoid receptor (MR) demonstrated that the ancestral molecule associated with ligands similar to those bound by the modern MR (Ortlund et al., 2007). Thus, at least in this branch of the NR3 subfamily, ligand binding was plesiomorphic and not acquired later in molecule evolution.

In summary, although their existence awaits confirmation, it appears likely that NHR ligands will turn out to play important roles in many aspects of *C. elegans* biology. Given the multitude of processes that are influenced by *C. elegans* NHRs (development, nutritional regulation, etc.), it appears probable that NHR ligands will be as diverse. A subset of NHRs may display a low-affinity ligand:NHR interaction akin to fatty acids binding to HNF4, whereas others, as exemplified by the DA:DAF-12 association, may mimic the high-affinity characteristics typically associated with steroid receptors. Across this spectrum, through study of the NHR family expansion, we stand to gain important insight into the evolution of both ligands and ligand binding, and how they impact transcriptional regulatory networks.

6. Conclusions and outlook

In the decade since the unveiling of the *C. elegans* genome, much progress has been made to understand the functions of nematode NHRs. Clearly significant frontiers remain, as we are far from understanding the biological roles of these unique regulators in nematodes–one of the most abundant metazoan genera on earth. *C. elegans* and other nematodes provide excellent opportunities to gain new insights into NHR evolution. In addition to their status as an excellent genetic, genomic, and pharmacological model organism, nematodes are suitable for whole animal biochemical analysis, which combined with immunopurification-mass spectrometry can shed light on both interacting proteins and cognate ligands. Recent advances in genomics promise to reveal genomic actions, and the ever-increasing number of RNAi screens in *C. elegans* can identify new roles of uncharacterized NHRs. The nematode NHR family expansion may offer refined focus and new insight into the functional interactions of cofactors, response elements and ligands with receptors, as well as a powerful window into the evolution of NHR-governed transcription networks.

Note added in proof

While this article was in press, Mullaney et al. reported that the LBD of NHR-25 can accommodate phosphoinositide lipids, suggesting that these molecules may represent an additional class of ligands for C. elegans NHRs.

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