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Functional modularity of nuclear hormone receptors in a Caenorhabditis elegans metabolic gene regulatory network

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Gene regulatory networks (GRNs) provide insights into the mechanisms of differential gene expression at a systems level. GRNs that relate to metazoan development have been studied extensively. However, little is still known about the design principles, organization and functionality of GRNs that control physiological processes such as metabolism, homeostasis and responses to environmental cues. In this study, we report the first experimentally mapped metazoan GRN of Caenorhabditis elegans metabolic genes. This network is enriched for nuclear hormone receptors (NHRs). The NHR family has greatly expanded in nematodes: humans have 48 NHRs, but C. elegans has 284, most of which are uncharacterized. We find that the C. elegans metabolic GRN is highly modular and that two GRN modules predominantly consist of NHRs. Network modularity has been proposed to facilitate a rapid response to different cues. As NHRs are metabolic sensors that are poised to respond to ligands, this suggests that C. elegans GRNs evolved to enable rapid and adaptive responses to different cues by a concurrence of NHR family expansion and modular GRN wiring.

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Introduction

The differential expression of metazoan genes in space and time is of critical importance to many biological processes. Genes need to be turned on and off at specific developmental time points to instruct processes such as organogenesis and morphogenesis. Differential gene expression is, at the first level, carried out by transcription factors (TFs) that control the expression of their target genes by physically interacting with cis-regulatory DNA sequences, such as promoters and enhancers. Of the 20 000 genes in a metazoan genome, 5–10% encode TFs, and these TFs occur in families that share similar types of DNA-binding domains (Reece-Hoyes et al, 2005; Vaquerizas et al, 2009). TFs can interact with and regulate large numbers of genes, and, conversely, some genes can be regulated by large numbers of TFs (Harbison et al, 2004; Deplancke et al, 2006a). Altogether, interactions between genes and their transcriptional regulators can be graphically represented in gene regulatory network (GRN) models, in which regulatory proteins and targets are represented as nodes, and the interactions between them are indicated as edges (Davidson et al, 2002; Walhout, 2006; Arda and Walhout, 2010). GRN models can provide insights into the mechanisms of transcriptional regulation at a systems level by connecting global and local network organization to network functionality. For instance, network modules and motifs can be used to explain the dynamics and organizing principles of gene regulation (Milo et al, 2002; Segal et al, 2003; Vermeirssen et al, 2007a; Martinez et al, 2008).

So far, GRN studies have extensively focused on unicellular organisms such as bacteria and yeast, and, to a lesser extent, on GRNs involved in metazoan development (Harbison et al, 2004; Davidson and Levine, 2005; Resendis-Antonio et al,
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2005; Sandmann et al, 2007). However, surprisingly little is known about the GRNs that control different aspects of systems physiology, even though differential gene expression likely continues to have an important function in post-developmental processes. Indeed, numerous human diseases, including obesity, diabetes and cancer are characterized by profound changes in gene expression.

\textit{Caenorhabditis elegans} provides a powerful model organism to study metazoan GRNs. It is genetically tractable, its development and lineage have been extremely well characterized and numerous resources are available that enable systematic genomic studies of gene expression (Reboul et al, 2003; Dupuy et al, 2004). Several GRNs have been characterized to various degrees in \textit{C. elegans}. These pertain to protein-coding gene sets of endoderm, digestive tract, neurons, the C-lineage and the vulva, as well as microRNA and bHLH TF-encoding genes (Maduro and Rothman, 2002; Baugh et al, 2005; Deplancke et al, 2006a; Vermeirssen et al, 2007a; Martinez et al, 2008; Ritie et al, 2008; Grove et al, 2009). Despite these efforts, however, little is known about the networks that control systems level, post-developmental gene expression in the nematode. Most TFs are expressed throughout the lifetime of the animal (Hunt-Newbury et al, 2007; Reece-Hoyes et al, 2007; Grove et al, 2009), strongly suggesting that differential gene expression is as important in post-developmental processes as it is during development. For instance, \textit{C. elegans} can respond to nutrient availability in its environment; in laboratory settings, it feeds on bacteria and exhibits a starvation response on food withdrawal that is correlated with major changes in gene expression (Van Gilst et al, 2005a; Baugh et al, 2009).

Nuclear hormone receptors (NHRs) are well-known regulators of different aspects of systems physiology, including endocrine signaling and metabolism (Chawla et al, 2001). Two well-studied \textit{C. elegans} NHRs include DAF-12, a vitamin D receptor homolog (Antebi et al, 2000), and the HNF4 homolog NHR-49, which has an important role in fat metabolism and in the starvation response (Van Gilst et al, 2005a, b). Remarkably, the \textit{C. elegans} genome encodes 284 NHRs, whereas humans have only 48 and \textit{Drosophila} 18 (Maglich et al, 2001). Most \textit{C. elegans} NHRs (269) are homologs of HNF4, of which there are two variants in humans and only one in \textit{Drosophila} (Palanker et al, 2009). In humans, HNF4\textalpha mutations lead to an early onset diabetic disorder, maturity onset diabetes of the young (MODY1) (Yamagata et al, 1996). In \textit{Drosophila}, dHNF4 mutants are sensitive to starvation and store higher levels of fat, suggesting that dHNF4 responds to nutrient availability (Palanker et al, 2009). Thus, HNF4 likely have an important function in post-developmental, metabolic GRNs in humans and flies. So far, only few \textit{C. elegans} NHRs have been characterized, and for most their physiological and molecular functions remain unknown. Furthermore, the evolutionary advantages of NHR family expansion have remained elusive, and the organization and functionality of \textit{C. elegans} NHRs in the context of GRNs remain completely uncharacterized.

NHRs interact with ligands to regulate their target genes (Chawla et al, 2001; Magner and Antebi, 2008). For instance, PPARs respond to fatty acids, and LXR, FXR, SXR and CAR are receptors for sterols, bile acids and xenobiotics, respectively (Chawla et al, 2001). Thus, NHRs likely function as metabolic sensors to rapidly respond to endogenous or exogenous signals (Magner and Antebi, 2008). In \textit{C. elegans} only a single NHR ligand has been identified: dafachronic acid, which interacts with and regulates DAF-12 activity (Motola et al, 2006).

Upon binding to their genomic sites, NHRs nucleate the assembly of multifactor transcriptional regulatory complexes by recruiting gene- and cell-specific cofactors. In mammals, these include PGC-1 cofactors and members of the Mediator complex, such as MED1 and MED15 (Lin et al, 2005; Yang et al, 2006; Li et al, 2008a; Naar and Thakur, 2009). In \textit{C. elegans}, DIN-1 functions as a cofactor for DAF-12, and MDT-15, the MED15 ortholog, interacts with NHR-49 and the SREBP-1 ortholog, SBP-1 (Taubert et al, 2006; Yang et al, 2006). MDT-15 has a broader role in metabolic gene expression than these two partners, suggesting that it interacts with additional TFs (Taubert et al, 2006, 2008).

GRNs can be experimentally delineated using either TF-centered (‘protein-to-DNA’) or gene-centered (‘DNA-to-protein’) methods (Arda and Walthout, 2010). TF-centered methods such as chromatin immunoprecipitation have been extremely powerful in yeast and in relatively uniform tissue-culture systems (Harbison et al, 2004; Kim and Ren, 2006). Studies of more complex systems such as whole organs, tissues or animals, however, have been limited to the analysis of one or a handful of TFs at a time (Odom et al, 2006; Sandmann et al, 2007). Gene-centered methods have classically used reporter gene assays, for instance to delineate early developmental gene expression in the sea urchin (Davidson et al, 2002). However, these methods are difficult to apply to larger sets of genes. We have previously used Gateway-compatible yeast one-hybrid (Y1H) assays for gene-centered GRN studies. This system can be used with dozens of genes at a time to rapidly retrieve multiple TFs in a condition-independent manner (Deplancke et al, 2004, 2006a; Vermeirssen et al, 2007a; Martinez et al, 2008). Thus, it is particularly suitable for delineating GRNs that pertain to different sets of genes, such as those involved in different aspects of systems physiology.

In this study, we present the first experimentally mapped, metabolic GRN in a metazoan model system. This network contains hundreds of protein–DNA interactions between a set of metabolic genes and numerous TFs. We find that the GRN is enriched for NHRs compared with other gene-centered networks and that it is highly modular. Two modules mainly contain NHRs, and, remarkably, most of these NHRs confer a metabolic phenotype. Network modularity has been proposed to facilitate rapid and robust responses to environmental cues (Babu et al, 2004). Together these observations indicate that NHR family expansion concurs with adaptive wiring of nematode GRNs. We also identify new interactions between MDT-15 and 12 TFs, and find that these TFs are enriched for NHRs that occur in the GRN, illustrating the central role of this mediator component in metabolic gene regulation. On the basis of our results, we propose a model for the evolution and organization of \textit{C. elegans} metabolic GRNs.
Results

A gene-centered GRN of *C. elegans* metabolic genes

To gain insight into the organization and functionality of GRNs involved in systems physiology, we first selected a set of genes that have been implicated in *C. elegans* metabolism. Two thirds of this set was identified in a genome-wide RNAi screen for animals with an altered Nile Red staining pattern in multiple genetic backgrounds (Ashrafi et al., 2003). When used as a vital dye, Nile Red stains ‘fat-containing lysosome-like organelles’ in the *C. elegans* intestine (Schroeder et al., 2007; Rabbits et al., 2008). Thus, the genes uncovered in the RNAi study may be involved in lipid metabolism, and/or in other types of metabolism such as the general catabolism of biomolecules.

The other third of our gene set was identified in an effort to find metabolic genes whose expression is affected by food availability. These ‘fastering response genes’ give a robust transcriptional response upon short-term food withdrawal, and the regulation of some, but not all of these, is dependent on the nuclear receptor, NHR-49 (Van Gilst et al., 2005a). Hereafter, these genes will collectively be referred to as ‘metabolic genes’ (Supplementary Figure S1).

To identify proteins that can interact with metabolic genes, we cloned the promoters of 71 metabolic genes upstream of the Y1H reporter genes HIS3 and LacZ, and integrated the resulting promoter::reporter constructs into the yeast genome to create Y1H ‘bait’ strains (Deplancke et al., 2004, 2006b) (Supplementary Table S1). We screened each bait strain versus a cDNA library (Walhout et al., 2000b), and a TF mini-library (Deplancke et al., 2004). Subsequently, we tested each bait strain versus each TF identified, both to retest interactions and to identify additional ones that were missed in the library screens (Supplementary Table S2). We then scored and filtered the Y1H interactions as described to minimize the inclusion of false positives (Vermeirssen et al., 2007a). Finally, we used Cytoscape (Shannon et al., 2003) to combine all interactions into a GRN graph model (Supplementary Table S3; Figure 1A).

In total, the metabolic GRN contains 508 interactions between 69 metabolic gene promoters and 100 TFs (Figure 1A). The network is densely wired and the overall structure is similar to that of other gene-centered GRNs (data not shown) (Deplancke et al., 2006a; Vermeirssen et al., 2007a; Martinez et al., 2008). All components are connected in a single graph because of the presence of both highly connected promoters and highly connected TFs. However, we did observe a striking difference: more than a quarter of the TFs retrieved here are NHRs, which is significantly more than in the digestive tract, neuronal and microRNA networks (Deplancke et al., 2006a; Vermeirssen et al., 2007a; Martinez et al., 2008) (Figure 1B). This is exciting because, as mentioned above, NHRs can function as metabolic sensors. The retrieval of many NHRs suggests that the expansion of this family relates to metabolic functionality. The difference with the digestive tract network is relatively modest ($P \approx 0.05$), which is probably because the intestine is the most important metabolic tissue in *C. elegans*. Overall, 41 of the 69 promoters (~60%) interacted with one or more NHR, suggesting that the promoters of *C. elegans* metabolic genes may have an inherent preference for NHRs, and that multiple NHRs may regulate metabolic gene expression.

NHRs organize into functional modules

Systems-level GRNs can capture hundreds of interactions between numerous genes and their regulators, and such networks are often too complex for manual analysis. Instead, mathematical and computational methods can be used to investigate the design principles and organization of GRNs. These principles can then be related to biological functionality. For instance, GRNs can be decomposed into ‘modules’; highly interconnected network neighborhoods consisting of nodes with similar functions (Ravasz et al., 2002; Vermeirssen et al., 2007a). Such modular network organization has been proposed to increase the adaptability of a system and to allow rapid and robust informational flow through the network (Ravasz et al., 2002; Babu et al., 2004).

To examine whether the *C. elegans* metabolic GRN has a modular architecture we performed topological overlap coefficient (TOC) analysis (Vermeirssen et al., 2007a). For each TF pair, we calculated a TOC score based on the number of target genes they share in the metabolic GRN, and clustered the TFs with similar TOC scores to identify TF modules. After TOC clustering, we found that the metabolic GRN is highly modular as it contains five TF modules (I–V) (Figure 1C). This is more than we have observed previously; the neuronal network consisted of only two TF modules, whereas the microRNA network did not contain any (Vermeirssen et al., 2008; data not shown). Interestingly, ~60% (~16 of 27) of all NHRs in the network are located in either one of two modules (modules II and III), and each of these modules consists predominantly of NHRs (66% each; Supplementary Table S4; Figure 1D; Supplementary Figure S2).

The observation that NHRs are wired into GRN modules that share target genes leads to the prediction that either (1) one or few of them are involved in metabolic regulation in *vivo*, (2) they all act redundantly, or (3) they all function in the regulation of systems physiology. The majority of the target genes of the NHRs that participate in modules have a metabolic phenotype as judged by an increase or decrease in Nile Red staining (Ashrafi et al., 2003). Thus, we performed systematic Nile Red staining on reduction of the activity of different NHRs by RNAi. Several NHRs in module II are essential for *C. elegans* development (Kamath et al., 2003) and could therefore not be examined. Nonetheless, RNAi of one NHR in module II and most NHRs in module III resulted in an increase in Nile Red staining (Figure 2A and B; Supplementary Figure S3). To further analyze changes in fat depots, we performed Oil-Red-O staining of animals subjected to nhr(RNAi). We found that RNAi of most NHRs resulted in increased Oil-Red-O staining, indicating that most of these NHRs indeed regulate fat storage or catabolism (Figure 3A and B). In *Drosophila*, the single HNF4 homolog is responsible for the regulation of fat storage (Palanker et al., 2009). In contrast, our findings indicate that, in *C. elegans*, multiple HNF4-type NHRs share this function, even after duplication and divergence. Altogether these findings show that module III contains functionally related NHRs that all regulate *C. elegans* physiology. The fact that the NHRs in module III are dispensable for development suggests that this function in post-developmental physiology, for instance to respond to...
environmental or dietary cues, whereas the NHRs in module II may regulate metabolic gene expression during development.

**MDT-15 specifically interacts with NHRs that occur in the metabolic GRN**

NHRs regulate gene expression by interacting with different transcriptional cofactors. In *C. elegans*, the HNF4 homolog NHR-49 interacts with the mediator component MDT-15 (Taubert et al, 2006). However, MDT-15 likely interacts with additional TFs to exert its broad metabolic functions. We performed comprehensive yeast two-hybrid (Y2H) assays using MDT-15 as bait versus our array of 755 full-length *C. elegans* TFs (80% of all 940) (Figure 4A) (Reece-Hoyes et al, 2005; Vermeirssen et al, 2007b). We did not have a functional clone for SBP-1, a known MDT-15 partner, (data not shown), but did confirm the interaction between MDT-15 and NHR-49 (Supplementary Table S5). Additionally, we identified 12 novel interactions between MDT-15 and *C. elegans* TFs (Figure 4A; Supplementary Table S5). MDT-15 has a central role in systems physiology. Therefore, we predicted that the TFs with which MDT-15 interacts should occur in our network. Indeed, statistical analysis of the TFs that interact with MDT-15 revealed three distinct features: they are significantly enriched for NHRs, for TFs that confer a Nile Red staining phenotype by RNAi and for TFs that occur in the metabolic GRN (Figure 4B; Supplementary Table S5). This confirms the prediction that MDT-15 interacts with many TFs and provides support for its central role in metabolic gene expression networks (Taubert et al, 2006).

The expression of the *C. elegans* short-chain acyl-CoA dehydrogenases acdh-1 and acdh-2 requires MDT-15, but not its partner NHR-49 (Taubert et al, 2006). In this study, we identified NHR-10 as the single NHR that can bind to the promoters of both acdh-1 and acdh-2 (Pacdh-1 and Pacdh-2) (Supplementary Table S3). In addition, NHR-10 can interact...
An integrated NHR network

We visualized all NHR–promoter interactions that contribute to modules II and III, the protein–protein interactions involving MDT-15, as well as the metabolic phenotypes in a single, integrated NHR network (Figure 5). We also included previously identified protein–protein interactions between NHR-49 and any NHR that is present in the network (Vermeirssen et al., 2007b). Several observations can be made from this GRN. First, MDT-15 interacts with NHRs from both modules II and III, and also interacts with TFs that receive inputs from either module. This further emphasizes the central role of MDT-15 in the regulation of metabolic gene expression. Second, three NHRs in module III can physically interact with NHR-49 (Vermeirssen et al., 2007b), and RNAi of any of them results in a fat storage phenotype, suggesting that NHR-49 shares different partners for its metabolic functions. Third, there are numerous interactions between NHRs and NHR-encoding genes, and several nhr gene promoters receive input from multiple other NHRs. For instance, Pnhr-49 interacts with four NHRs, both from modules II and III. This ‘interregulation’ implies that NHRs may function in transcriptional cascades to execute appropriate gene expression programs, for example, on receiving intrinsic or extrinsic signals (Magner and Antebi 2008). Fourth, several NHRs interact with multiple promoters,
which may ensure a coordinated response of these target genes. Fifth, there are multiple feed-forward loops in the network, in which an NHR controls another NHR, and both of these share a downstream target. Such loops likely enable a controlled signal response, and protect against fluctuations in gene expression (Alon, 2007). Finally, NHR-178 and NHR-86 can interact with their own promoters, suggesting that they may be auto-regulators.

NHR-86: a metabolic TF with high-flux capacity

The HNF4 homolog NHR-86 (module III) has a high-flux capacity (Martinez et al., 2008): its promoter is bound by 12 NHRs and it interacts with 7 gene promoters. In addition, NHR-86 interacts with MDT-15 and with its own promoter. We obtained the nhr-86(tm2590) mutant allele that carries a deletion removing part of exon 4 and intron 4 of the nhr-86 gene (Figure 6A). We raised a polyclonal antibody against NHR-86, and assayed total protein extracts from wild-type and nhr-86(tm2590) animals. We did not detect any NHR-86-specific protein in the mutant, even though the allele is predicted to produce a truncated protein (Figure 6B). It is possible that a truncated protein is expressed at very low levels, or in only a small subset of cells, and can therefore not be detected by western blotting of whole animal extracts. Nonetheless, we found that, though wild-type NHR-86 can bind to its own promoter in Y1H assays, truncated NHR-86 cannot (Figure 6C), even though both forms of NHR-86 are stably expressed in yeast (Supplementary Figure S4). Therefore, we conclude that tm2590 is likely a null allele of nhr-86. The observation that NHR-86 interacts with its own promoter in Y1H assays suggests that it may have auto-regulatory activity. To test this, we created transgenic animals expressing GFP under the control of Pnhr-86 and examined GFP expression in wild-type and nhr-86(tm2590) mutant animals. Compared with wild-type Pnhr-86 GFP animals, we found that GFP expression was substantially upregulated in the pharynx and hypodermis in 100% of the nhr-86(tm2590) mutant animals (Figure 6D). This suggests that NHR-86 is an auto-repressor in these tissues. Negative auto-regulation is an important feature of regulatory circuits; it accelerates the response to outside signals and promotes transcriptional robustness (Alon, 2007). We tagged the full-length nhr-86 ORF with GFP and generated transgenic animals, and found that NHR-86 predominantly localizes to the nuclei of intestinal and excretory gland cells, as well as several head neurons, which are all important tissues for metabolism and homeostasis (Figure 6E). We also found that nhr-86(tm2590) mutants exhibit an increase in Nile Red staining (data not shown) and Oil-Red-O staining, confirming the nhr-86 RNAi result. This phenotype was rescued by wild-type nhr-86 tagged with GFP (Figure 6F and G). Altogether our results show that NHR-86 is involved in lipid storage and/or catabolism.
NHR-178 and NHR-45 respond to nutrient availability

The expansion of NHRs, their modular wiring in the network and the observation that they all contribute to systems physiology suggest that these NHRs may respond to physiological or environmental cues such as nutrient availability. To further assess this, we focused on nhr-178, one of the target genes in module III. Reduction of nhr-178 by RNAi results in an increase in stored lipids (Figures 2 and 3). We created a transgenic C. elegans strain that expresses GFP under the control of Pnhr-178, and found that this promoter drives expression in the pharynx and the first anterior intestinal cells (Int1) (100 ± 0% of the animals) (Figure 7A). Interestingly, though there is no GFP expression in the hypodermis under well-fed conditions, GFP expression was specifically upregulated in this tissue on food withdrawal (84 ± 1% of the animals) (Figure 7A). When we provided starved animals with food, GFP expression in the hypodermis rapidly disappeared (Figure 7A). This suggests that nhr-178 functions in a gene circuit that responds to nutrient availability. NHR-178 participates in several transcriptional circuits that involve additional NHRs (Figure 5). One of these is NHR-45 for which we obtained an nhr-45(tm1307) mutant that carries a large deletion in the DNA-binding domain. This mutant recapitulates the Oil-Red-O staining profile observed with nhr-45 RNAi (Supplementary Figure S5A and B; Figure 3). To investigate the interaction between NHR-45 and Pnhr-178 in vivo, we crossed the nhr-45(tm1307) mutant into the Pnhr-178::GFP transgenic animals. Under well-fed conditions, loss of nhr-45 greatly reduced GFP expression in the pharynx and eliminated it in the Int1 cells (100 ± 0%) (Figure 7A). Microinjection of a wild-type nhr-45 construct restored the intestinal and pharyngeal GFP expression (80 ± 1% of the animals), showing that NHR-45 activates the promoter of nhr-178 in these tissues (Supplementary Figure S5C). Interestingly, however, in a small but reproducible number of nhr-45(tm1307) mutant animals (3.5 ± 0.7%) Pnhr-178 activity was upregulated in the hypodermis under well-fed conditions, mimicking the starvation response despite the presence of food (Figure 7A). When we challenged nhr-45(tm1307) mutants with food removal, Pnhr-178 activity was turned on in the hypodermis, which is similar to the response observed with wild-type background. In contrast, the mutants failed to completely suppress hypodermal Pnhr-178 activity when they were provided with food after starvation (90 ± 0.3% of the animals still exhibited hypodermal expression) (Figure 7A). Taken together, these observations reveal a gene circuit that involves multiple NHRs, which function to rapidly respond to nutrient availability. In this circuit, Pnhr-178 activity is kept off in the hypodermis under well-fed conditions. When animals starve, NHR-45 may be repressed, thereby allowing other TFs, again potentially NHRs, to activate hypodermal nhr-178 expression (Figure 7B).

Discussion

In this study, we present the first metazoan GRN pertaining to genes involved in systems physiology. This network provides...
novel insights into the design principles of network organization and how this relates to network functionality. Altogether, our observations suggest that *C. elegans* physiological GRNs evolved to enable rapid and robust responses to environmental or physiological cues by a concurrent of NHR family expansion and modular GRN wiring (Figure 8; see also below).

The metabolic network is not yet complete. First, we used only a subset of metabolic genes. Second, we have so far exclusively focused on gene promoters and it is likely that other cis-regulatory elements such as enhancers may be involved in differential metabolic gene expression as well. Third, not all interactions are detectable by Y1H assays; that is TFs for which we do not have a (correct) clone or that are underrepresented in the cDNA library will not be retrieved, and the system is not yet adapted to identify TF heterodimers. This may explain, at least in part, why we did not retrieve NHR-49, which dimerizes with more than 20 other NHRs (Vermeirssen *et al.*, 2007b) (see also below).

In addition to missing interactions, the network may also contain interactions that are not biologically relevant. These may be false positives, or alternatively do occur in *vivo*, but do not lead to an observable biological consequence. Indeed, it

**Figure 6** Analysis of NHR-86. (A) Gene model of nhr-86 indicating the tm2590 deletion (red rectangle), DNA-binding domain (green), and ligand-binding domain (blue). (B) Western blot using an anti-NHR-86 antibody showing that nhr-86(tm2590) mutant animals do not produce full-length NHR-86 protein (46 kDa, arrow); total protein extracts from wild-type (lane 1), or nhr-86(tm2590) mutant (lane 2) animals were used; (asterisk) non-specific band. (C) Mutant NHR-86 protein fails to bind *Pnhr-86* in Y1H assays: growth on permissive media (top), HIS3 reporter (middle), and LacZ reporter expression (bottom). (D) *Pnhr-86* activity determined by GFP expression in wild-type (top) or nhr-86(tm2590) mutant animals (bottom), indicating auto-repression by NHR-86 in head hypodermis and in the pharynx (white arrowheads). (E) Expression pattern and subcellular localization of NHR-86 as shown by GFP expression in transgenic animals carrying a *Pnhr-86::nhr-86::GFP* reporter construct. (F) Oil-Red-O staining of wild-type, nhr-86(tm2590), and nhr-86(tm2590) animals expressing the wild-type transgene (rescue). Black arrowheads point to the posterior bulb of the pharynx. (G) Quantification of Oil-Red-O staining shown in (F). *nhr-86* (tm2590) animals accumulate more intensely stained lipid droplets, and this phenotype is rescued by the wild-type nhr-86 gene (see Figure 3 for a description of the graph axes).
has been observed that TFs can interact with genomic locations without an apparent regulatory effect (Zeitlinger et al., 2007; Li et al., 2008b). Furthermore, it may be that interactions do have a biological effect but that this cannot be detected in vivo (i.e. it could be a false negative of the validation assay). For example, interactions that occur in a few cells, only under specific environmental conditions, or with small regulatory effects may be difficult to detect. Finally, it is

Figure 7  An NHR gene circuit that responds to nutrient availability. (A) Images showing Pnhr-178 activity in wild-type and nhr-45(tm1307) animals under different feeding conditions. Graphs next to each group of images show the percentage of animals that exhibit GFP expression in the indicated tissue/cells. Error bars indicate the s.e.m. Top panels—Nomarski images; bottom panels—GFP fluorescence. White arrowheads—first anterior intestinal cells (Int1); yellow arrowheads—hypodermis. (B) Cartoon depicting effects of NHR-45 and different feeding states on Pnhr-178 activity.
of course also possible that some interactions that we retrieved really do not occur in vivo and are actual false positives. We have aimed to minimize the inclusion of false interactions in our network by ensuring the technical quality of our experiments; the DNA baits are integrated into the yeast genome and are, therefore, both present at fixed copy number and in a chromatinized state. This is essential to avoid spurious interactions (Deplancke et al., 2004). In addition, we scored and filtered all our Y1H data to minimize the inclusion of ‘sticky’ TFs or interactions that were retrieved with highly auto-active promoters (Vermeirssen et al., 2007a). Despite some of these potential limitations, the results presented here, and in our other studies, show the use of Y1H assays to identify meaningful TF–target gene interactions (Deplancke et al., 2004, 2006a; Vermeirssen et al., 2007a, b; Martinez et al., 2008; Reece-Hoyes et al., 2009). Most importantly, the Y1H system uniquely enabled us to delineate a GRN pertaining to physiology because it can be used with multiple genes to retrieve multiple TFs. Indeed, more than 10% of all predicted C. elegans TFs (Reece-Hoyes et al., 2005) were retrieved in our network.

Our data provide the first insights into the organizing principles of C. elegans GRNs that pertain to systems physiology (Figure 8). The metabolic GRN is enriched for NHRs compared with other, similarly mapped networks. NHRs are known regulators of metabolic gene expression and this enrichment, therefore, provides global support for the overall quality of our network. NHRs regulate physiological gene expression on interactions with small molecule ligands such as hormones and fatty acids (Chawla et al., 2001). Thus, NHRs can be viewed as metabolic sensors that rapidly respond to physiological or environmental cues, allowing them to quickly change the expression of their target genes. The C. elegans family of NHRs has greatly expanded compared with other animals, and this expansion only involves duplications of an ancestral HNF4 gene, of which there is one in flies and two in humans, but 269 in C. elegans. Interestingly, the remarkable abundance and diversity of NHRs have also been observed in other nematodes. The genomes of both C. briggsae (232 NHRs) and C. remanei (256 NHRs) encode comparable numbers of NHRs (Haerty et al., 2008). Although there is about 50% reciprocal orthology, the remaining half of these NHRs are unique to each species. This suggests that NHRs evolve more rapidly than other types of TFs, which may be important in the adaptation to different environmental needs.

The metabolic GRN is highly modular and most NHRs occur in two TF modules. Network modularity has been proposed to facilitate rapid and robust changes in gene expression (Ravasz et al., 2002; Babu et al., 2004). Previously, it has been shown that biochemical networks composed of reactions between metabolic enzymes and their substrates are modular as well (Ravasz et al., 2002). Thus, we propose that C. elegans has acquired modularity in multiple layers of its physiological networks (Figure 8).

Several NHRs in the modules have highly similar amino acid sequences, and may share target genes because they bind similar DNA motifs. However, the observation that RNAi of most of these NHRs confer a similar metabolic phenotype suggests that they are not simply redundant. Indeed, it has been shown that the delta-9 fatty acid desaturase gene, fat-7, is regulated by at least two NHRs: NHR-49 and NHR-80 (Brock et al., 2006). NHRs are expressed in a variety of tissues, including the intestine and hypoderms (Reece-Hoyes et al., 2007; Vermeirssen et al., 2007a; this study). Thus, it could be that NHRs respond to signals and regulate overlapping sets of target genes in different tissues or under different conditions. C. elegans is a pseudocoelomate with a simple alimentary

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Figure 8 Model for the organizing principles of C. elegans metabolic networks. Circles are NHRs, and hues of purple represent the evolution of HNF4 family NHRs in C. elegans. These NHRs are organized into TF modules in the metabolic GRN. NHRs regulate their metabolic target genes (blue diamonds) by interacting with ligands (hexagons), and with other proteins (orange lines) such as the cofactor MDT-15 (orange oval) and dimerization partners.
system composed of the pharynx, intestine and rectum. This is in contrast to larger animals such as flies and mammals that have specialized organs for digestion, detoxification and endocrine signaling such as liver, kidney and pancreas. *C. elegans* live in soil and need to rapidly respond to changes in food conditions and other cues such as toxins or pathogens. As NHRs directly interact with diffusible ligands, expansion of this family could result in an increase in the capacity of the animal to directly respond to different physiological or environmental cues, thereby providing an adaptive advantage.

We previously found that dimerization, spatiotemporal expression and target gene specificity all contribute equally to divergence in the *C. elegans* bHLH family of TFs (Grove et al., 2009). It is likely that differential interactions with specific ligands provide an additional layer of divergence for *C. elegans* NHRs. So far, only a single NHR ligand in *C. elegans* has been identified (Motola et al., 2006). Future experiments will uncover additional ligands and will reveal whether each NHR responds to a single ligand, or if there may be redundancy or even modularity at this level as well. In addition, protein–protein interactions will provide additional complexity. For instance, NHR-49 dimerizes with many NHRs, at least three of which have a fat storage phenotype when knocked down by RNAi. Many NHRs in the GRN interact with the transcriptional cofactor MDT-15. In the future, it will be important to integrate interactions involving microRNAs, RNA-binding proteins and kinases into physiological GRNs to further our understanding of the organizing principles of complex cellular networks and how these principles relate to network functionality and evolution.

### Materials and methods

#### Promoter cloning

Gene promoters were defined and cloned as described (Dupuy et al., 2004). Briefly, promoters were amplified from *C. elegans* (N2) genomic DNA by PCR, cloned into two *Y1H* reporter Destination vectors through Gateway cloning and integrated into the genome of *Saccharomyces cerevisiae* (YM4271) to create Y1H bait strains as described (Deplancke et al., 2006b). In total, 71 Y1H bait strains were successfully generated and screened in Y1H assays. Primer sequences and detailed information about Y1H bait strains are provided in Supplementary Table S1.

#### Y1H assays

Y1H assays were performed as described (Deplancke et al., 2006b; Vermeirssen et al., 2007a; Martinez et al., 2008). Two different prey libraries (AD-wrmcDNA, Walhout et al., 2006b) and AD-TF mini-library (Deplancke et al., 2004) were used to screen each Y1H bait strain to identify interacting TFs. All interactions were retested in fresh yeast by PCR/gap repair (Deplancke et al., 2006b). The ORFs of preys that retested were PCR amplified from corresponding yeast strains and were sequenced by Agencourt Bioscience Corporation. Interacting TFs were identified using the BLASTX algorithm. In total, 306 unique interaction sequence tags were obtained (Walhout et al., 2006a). Y1H matrix experiments were performed by transforming available prey clones of all interacting TFs obtained in the screens, and several TFs found earlier (Deplancke et al., 2006a; Vermeirssen et al., 2007a; Martinez et al., 2008) into each Y1H bait strain individually (174 preys were used in total; Supplementary Table S2). Each metabolic gene promoter–TF interaction was evaluated using a standardized and stringent Y1H scoring and filtering system (Vermeirssen et al., 2007a). Only interactions with a score ≥ 5 were considered (Supplementary Table S3). All interactions are available in the EDGEdb database (Barrasa et al., 2007).

#### TOC analysis

TOC analysis and clustering was performed as described (Vermeirssen et al., 2007a).

#### Y2H matrix assays

Y2H matrix assays were performed by mating as described (Walhout and Vidal, 2001; Vermeirssen et al., 2007b). The DB-MDT-15-NT construct (Taubert et al., 2006) was transformed into MaV103 yeast and used as bait. This bait strain was mated against *C. elegans* TF array (wTF2.1), which consists of 755 TFs (Vermeirssen et al., 2007b). Diploids were selected on permissive media (Sc-Leu-Trp) and subsequently replica plated onto selective media (Sc-His-Leu-Trp + 20 mM 3-amino-1,2,4-triazole) to assay for HIS3 reporter gene activity. Diploids that grew on these plates were picked and assayed for LacZ reporter gene activation. Only TFs that conferred positive read-outs in both reporter gene assays were considered as interacting TFs. The identities of the interacting prey TFs were determined based on their position in the array, and confirmed by sequencing.

### C. elegans strains and growth conditions

*C. elegans* N2-Bristol strain was used as wild type. For a complete list of strains used and generated in this study, see Supplementary Table S6. All strains were maintained at 20°C as described (Brenner, 1974). The alleles, *nhr-45(tm1307)* and *nhr-86(tm2590)* were generously provided by the National Bioresource Project, Japan. All deletion strains were out-crossed at least four times with N2.

#### Starvation experiments

The assays were done as described (Van Gilst et al., 2005a, b). Briefly, animals were collected and washed several times with M9 buffer to remove residual bacteria. The animals were then plated onto nematode growth medium (NGM) that did not contain food, and allowed to fast on these plates for 12 h at 20°C, and then scored for hypodermal GFP expression. After the starvation period, animals were transferred back on NGM plates with food and allowed to recover for 24 h at 20°C, and then scored for hypodermal GFP expression. The data reported in Figure 7A were obtained by using synchronized L1 stage animals for starvation assays. Similar results were obtained with L3 stage animals (data not shown).

#### RNAi experiments

We generated RNAi constructs for *nhr-86, nhr-234, nhr-109, nhr-273, nhr-41, nhr-178, nhr-79, nhr-12, nhr-28, nhr-70 and nhr-102* by transferring the full-length ORFs from ORFeome v3.1 into pL4440-Dest-RNAi (Rual et al., 2004) through Gateway cloning. Additional RNAi clones were cherry picked from the *C. elegans* RNAi library (Kamath et al., 2003). HT115(DE3) bacterial strains carrying RNAi constructs were grown in Luria Broth containing ampicillin (50 µg/ml) at 37°C for 8–10 h until they reach OD600 ~ 1.0. Bacteria were pelletted by centrifugation, washed once with M9 buffer, resuspended in M9 buffer and seeded on NGM plates containing 5 mM IPTG and 50 µg/ml ampicillin. Bacteria were induced overnight at 22°C. The next day, either ~3 synchronized L1 larvae (for Nile Red staining) or five L4 larvae (for GFP expression analyses) were placed on the plates. All RNAi clones were verified by sequencing.

#### Vital Nile Red staining and quantification

Nile Red staining was performed as described (Ashraf et al., 2003). Briefly, Nile Red powder (N-1142 Molecular Probes, Invitrogen) was dissolved in acetone to make a 0.5 mg/ml stock solution, which was
kept at −20°C. This stock solution was then diluted 1:500 in 1× phosphate-buffered saline, and overlaid on top of NGM plates either followed by Dunnett’s Multiple Comparison post test, using GraphPad experiments of multiple NHRs was performed by one-way ANOVA, pixel intensities, we followed the procedure outlined in Srinivasan et al. (2008). Identities of images were masked while recording data to prevent observer bias. Statistical analysis of RNAi-coupled Nile Red experiments of multiple NHRs was performed by one-way ANOVA, integrated, or alternatively the best transmitting line, was selected we obtained up to 10 independent lines that all showed identical promoters used to generate the exception that C. elegans Molecular Systems Biology 2010

Oil-Red-O staining and quantification

Oil-Red-O staining was performed as described in Soukas et al. (2009).

Quantification of Oil-Red-O staining by image processing

RGB images of stage-synchronized, 1-day old adult animals (n=3) were acquired using identical bright field settings to keep background illumination constant across different samples. These images were digitally oriented and cropped to include only the anterior part of each worm, standardizing the area to be processed. The RGB composite images were then split into each contributing color channel: red, green and blue. Of the three, the green channel was selected to further analyze pixel intensities, as it gives the best contrast for the red Oil-Red-O stain. The images were inverted to make the background dark, and thresholded to separate stained areas from the background using Imagemagel software. The number of pixels corresponding to various pixel intensities from the thresholded areas was counted and plotted.

Microscopy

Nomarski and fluorescence images were obtained using a Zeiss Axioscope 2+ microscope. Images of GFP expression or Nile Red fluorescence were captured using a digital CCD camera (Hamamatsu C4742-95-12ERG) and Axiosvision (Zeiss) software. GFP fluorescence images were obtained using a FITC filter (excitation 460–500 nm, emission S10–S56 nm). Nile Red fluorescence images were taken using a rhodamine filter (excitation S52–S555 nm, emission S573–S630 nm). Animals were placed into a drop of 0.1% sodium azide in M9 buffer on a rhodamine filter (excitation 525–555 nm, emission 575–630 nm). Images were obtained using a FITC filter (excitation 460–500 nm, emission S10–S56 nm). Nile Red fluorescence images were taken using a rhodamine filter (excitation S52–S555 nm, emission S573–S630 nm). Animals were placed into a drop of 0.1% sodium azide in M9 buffer on a fresh 2% agarose pad for observation. Oil-Red-O images were acquired using Zeiss AxioCam HRc color CCD camera.

Generation of promoter::GFP transgenic C. elegans and quantification of GFP expression

Transgenic animals were created by ballistic transformation into unc-119(ed3) animals as described (Reece-Hoyes et al., 2007), with the exception that Pnhr-86::nhr-86NT::GFP was cloned by multi-site Gateway LR reaction into pDEST-MB14 (Dupuy et al., 2004). The promoter sequences used to generate promoter::GFP constructs were identical to those used in Y1H assays. For each GFP construct we obtained up to 10 independent lines that all showed identical GFP expression patterns (data not shown). When available, an integrated, or alternatively the best transmitting line, was selected for subsequent experiments. GFP expression was scored for each genotype in at least 25 animals, and each experiment was repeated at least twice.

Genetic rescue experiments

The nhr-86 rescue strain was generated by crossing nhr-86(tm2590) mutants to the VLS05 strain, which carries the Pnhr-86::nhr-86NT::GFP construct (Supplementary Table S6). The nhr-45 rescuing fragment was PCR amplified using the forward primer CTCTTTATTAT GCATTTTGTGTTT and the reverse primer TCACCTGGAAAACGTGAGACT CA from C. elegans (N2) genomic DNA. This fragment consists of the genomic sequences 2 kb upstream of the translational start site of nhr-45, nhr-45 gene itself, and 467 bp downstream of the 3’end of nhr-45. The PCR band corresponding to the expected size was gel purified and sequence verified. Transgenic animals were generated by microinjecting 10 ng/µl of the purified PCR product along with 80 ng/µl of the coinjection marker plasmid pRF4, which carries the marker gene rol-6(su1006) into the germline of the VL739 strain (Supplementary Table S6) (Mello et al, 1991).

Immunoblotting

C. elegans total protein extract preparation

L4 stage animals were collected and washed with sterile water. Animals were homogenized by sonicating in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 1 mM DTT, and Roche Complete protease inhibitors). The lysates were cleared by centrifugation and protein concentrations were determined using Bio-Rad DC Protein Assay (cat. #: 500–0116). In all, 100 mg of total protein extract was loaded in each lane.

Yeast sample preparation

Yeast strains were grown in YEFP liquid medium at 30°C with a starting density of OD600 ~0.1 until they reach an OD600 ~0.6–0.8. For each sample, 1 ml of liquid culture was pelleted and washed with sterile water. The pellet was then resuspended in freshly made, ice-cold 150 mM NaOH solution. After incubating on ice for 15 min, the cells were pelleted, resuspended in 20 µl of 2× Laemmli buffer, boiled for 5 min and loaded in equal amounts onto the gel. In both cases, proteins were separated using NuPAGE 4–12% Bis–Tris gels (Invitrogen, cat #: NP0323), and transferred onto PVDF membranes. The membranes were incubated overnight at 4°C with primary antibodies, followed by standard immunoblotting techniques.

Antibodies
We raised a polyclonal anti-NHR-86(NT) antibody in rabbits using an N-terminal peptide (SQFRPEKEKSTCSIC, AnaSpec Inc., San Jose, CA), and used this at a final concentration of 1 mg/ml. For detecting Gal4AD fusions from yeast extracts, an anti-Gal4AD antibody from Sigma (cat #: G9293) was used.

Supplementary information
Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

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Conflict of Interest
The authors declare that they have no conflict of interest.
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