

# 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*,

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.

*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 *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; Ririe *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, *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 *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 *C. elegans* genome encodes 284 NHRs, whereas humans have only 48 and *Drosophila* 18 (Maglich *et al*, 2001). Most *C. elegans* NHRs (269) are homologs of HNF4, of which there are two variants in humans and only one in *Drosophila* (Palanker *et al*, 2009). In humans, HNF4 $\alpha$  mutations lead to an early onset diabetic disorder, maturity onset diabetes of the young (MODY1) (Yamagata *et al*, 1996). In *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 *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 *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 LXRs, 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 *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 *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 Walhout, 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 *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; Rabbitts *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 'fasting 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).

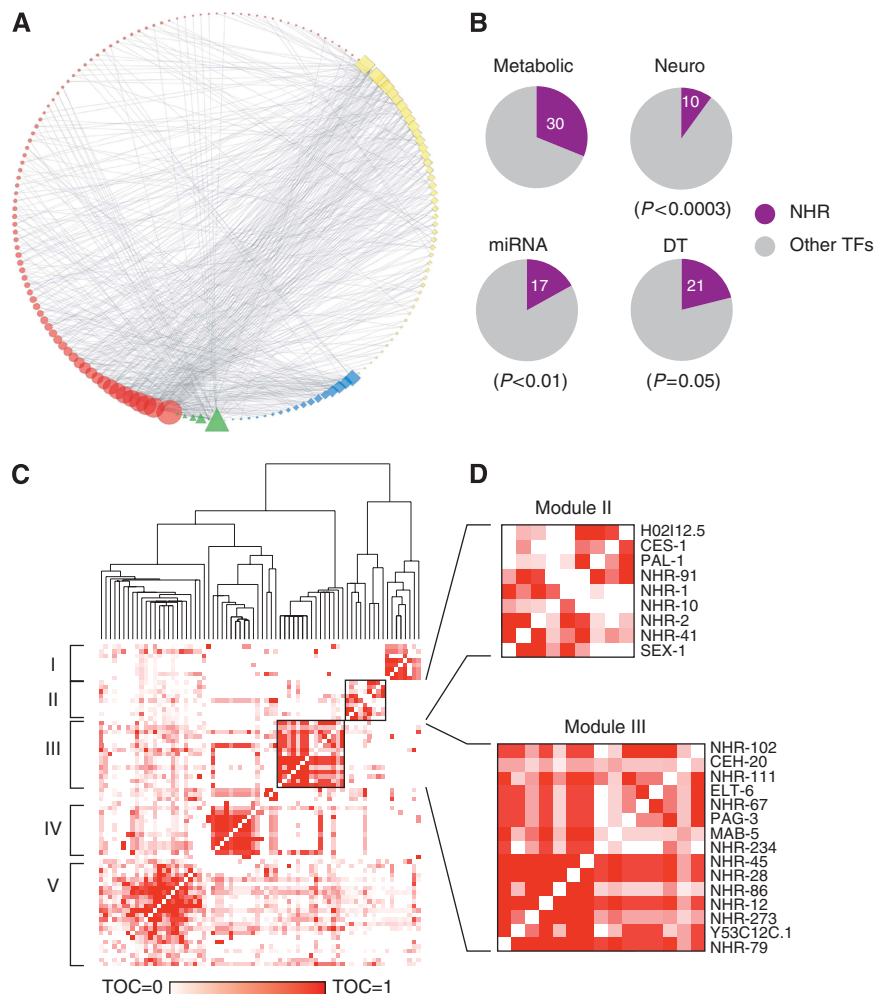
In total, the metabolic GRN contains 508 interactions between 69 metabolic gene promoters and 100 TFs (Figure 1A). The network is densely wired and the overall structure is similar to that of other gene-centered GRNs (data not shown) (Deplancke *et al.*, 2006a; Vermeirssen *et al.*, 2007a; Martinez *et 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 ( $P=0.05$ ), 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.*, 2007a; Martinez *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 these function in post-developmental physiology, for instance to respond to



**Figure 1** (A) *C. elegans* metabolic GRN. (A) Protein–DNA interaction network of *C. elegans* metabolic genes. Red circles—TFs; yellow diamonds—promoters of genes with Nile Red phenotypes; blue diamonds—promoters of fasting response genes; green triangles—TFs whose promoters were also analyzed; gray lines—protein–DNA interactions identified by Y1H assays. (B) Pie charts representing the percentage of NHRs in different gene-centered GRNs. Neuro—neuronal (Vermeirssen *et al*, 2007a); miRNA—microRNAs (Martinez *et al*, 2008); DT—digestive tract (Deplancke *et al*, 2006a). Putative novel TFs (that were specifically retrieved but that do not possess a known DNA-binding domain; Deplancke *et al*, 2006a) were omitted (two proportion z-test). (C) TOC clustering matrix of TFs in the metabolic gene network identifies GRN modules (roman numerals). The matrix is symmetrical across the white diagonal. Each cell that is at the intersection of two TFs represents the calculated TOC score for that TF pair. TOC scores are color coded as shown at the bottom of the panel. (D) Enlarged views of modules II and III, which predominantly contain NHRs. Source data is available for this figure at [www.nature.com/msb](http://www.nature.com/msb).

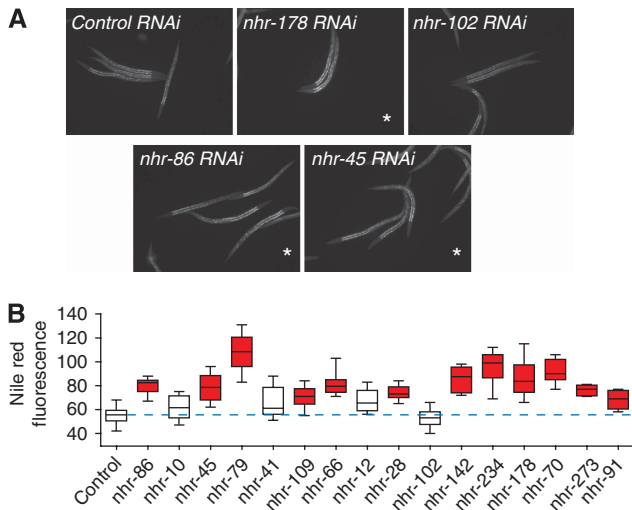
environmental or dietary cues, whereas the NHRs in module II may regulate metabolic gene expression during development.

### 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 *acd-1* and *acd-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 *acd-1* and *acd-2* (*Pacdh-1* and *Pacdh-2*) (Supplementary Table S3). In addition, NHR-10 can interact

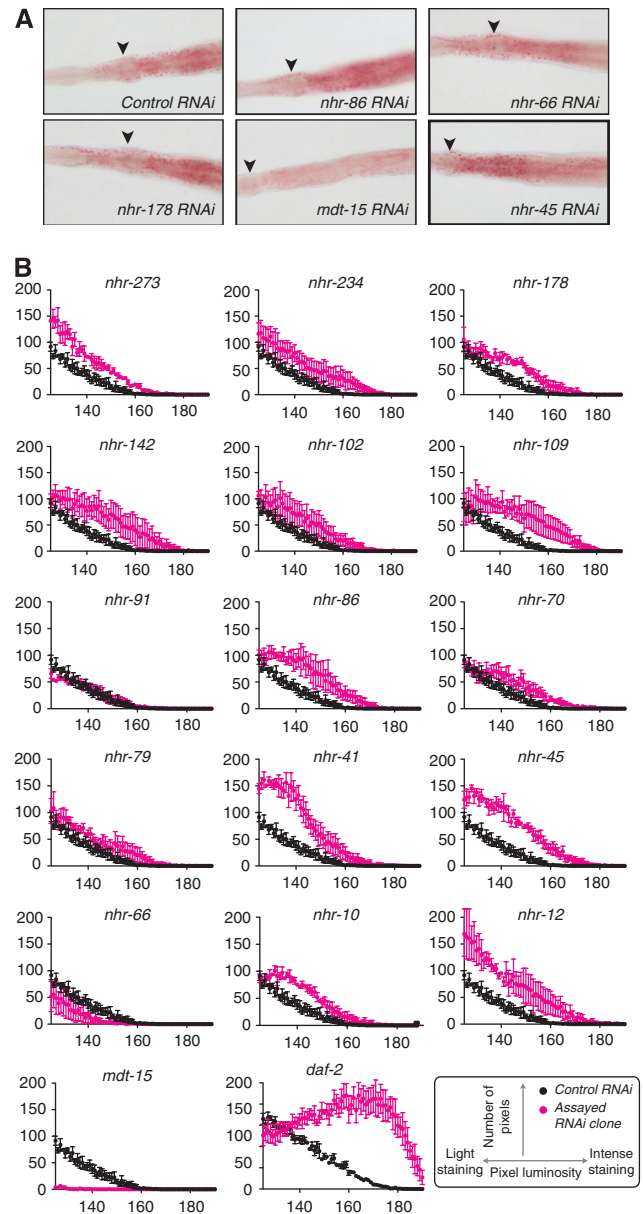


**Figure 2** Analysis of vital Nile Red staining of NHR knockdowns. **(A)** Examples of Nile Red phenotypes observed on NHR inactivation by RNAi. Asterisks indicate significant changes in Nile Red fluorescence compared with control animals (notice the increased fluorescent intensity). **(B)** Quantification of Nile Red staining-coupled RNAi experiments. Red box plots—statistically significant changes in Nile Red staining; white box plots—no significant change; dashed line—median Nile Red fluorescence in control RNAi animals. In each box plot, the central bar indicates the median, the edges of the box indicate the 25th and 75th percentiles, and the whiskers extend to the most extreme data points. For details of the statistical analysis, see ‘Materials and methods’.

with MDT-15 (Supplementary Table S5). To determine whether NHR-10 regulates *acdh-1* and/or *acdh-2* *in vivo*, we created transgenic animals expressing the green fluorescent protein (GFP) under the control of *Pacdh-1* or *Pacdh-2*, and knocked down either *mdt-15* or *nhr-10* by RNAi. We observed that RNAi of *mdt-15* greatly reduced GFP expression in the intestine of both *Pacdh-1::GFP* and *Pacdh-2::GFP* animals, confirming that these promoters are MDT-15 targets (Taubert et al, 2006) (Figure 4C and D). Importantly, knockdown of *nhr-10* conferred an identical *acdh* expression phenotype strongly suggesting that NHR-10 and MDT-15 together activate *acdh-1* and *acdh-2* expression by binding to their promoters *in vivo* (Figure 4C and D). Altogether, these data show that MDT-15 preferentially associates with NHRs that occur in the metabolic GRN to exert its functions.

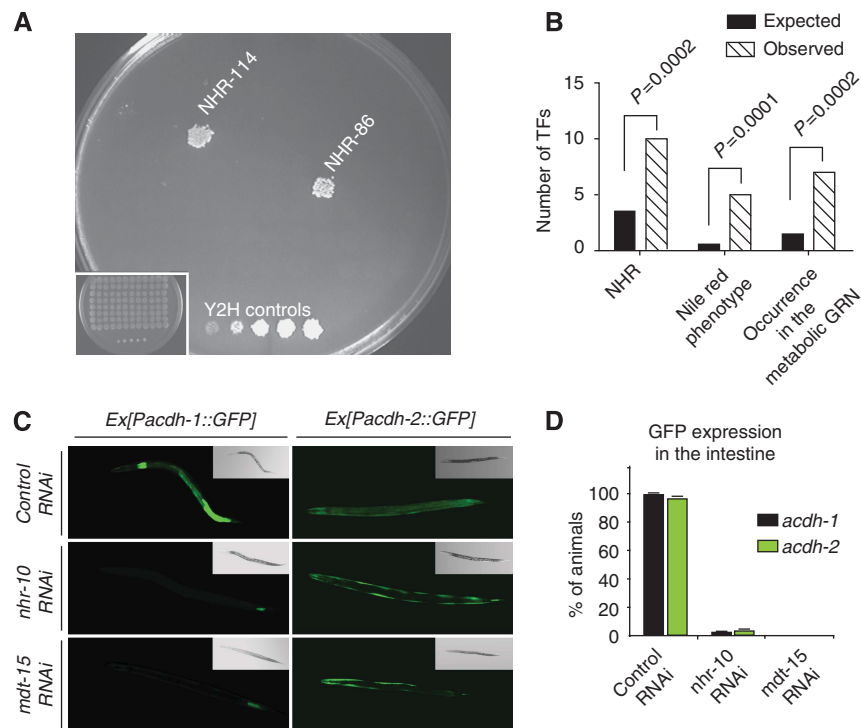
### An integrated NHR network

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



**Figure 3** Oil-Red-O staining analysis of NHR knockdown experiments. **(A)** Examples of Oil-Red-O stained animals. Anterior part of each animal is to the left. Black arrowheads point to the posterior bulb of the pharynx. **(B)** Quantifications of Oil-Red-O staining experiments. The inset at the bottom right of the figure describes graph axes. Error bars indicate the standard error of the mean (s.e.m.). The two graphs at the bottom are negative (*mdt-15 RNAi*) and positive controls (*daf-2 RNAi*).

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,



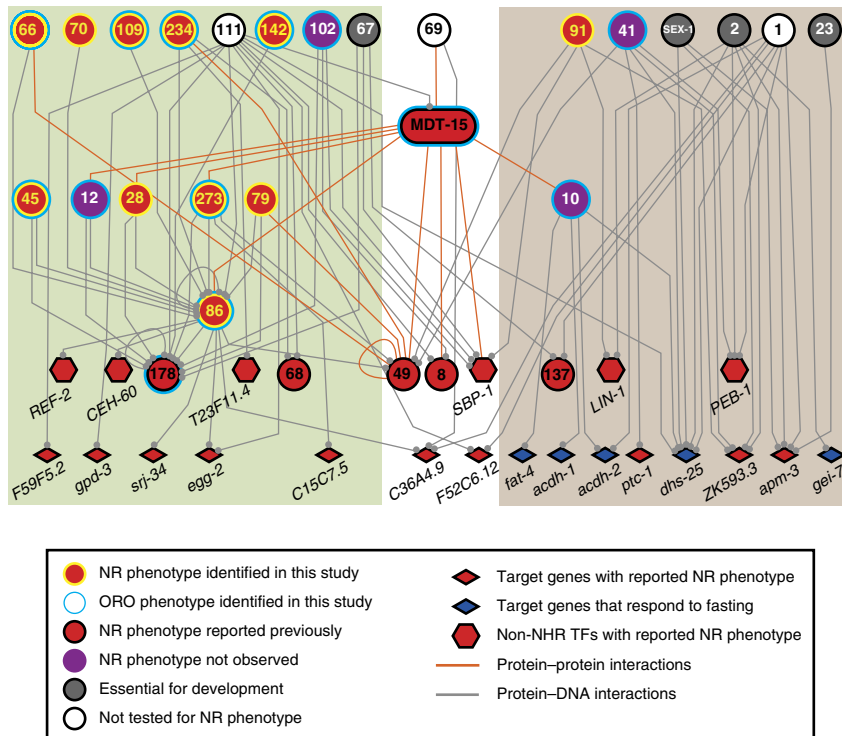
**Figure 4** MDT-15 specifically interacts with NHRs in the metabolic GRN. **(A)** Example of the Y2H matrix experiment using DB-MDT-15 bait. Indicated are the TFs that interact with MDT-15, which can grow on selective plates, inset—permissive plate. Five spots at the bottom are Y2H controls. **(B)** Bar graphs show the expected (solid bars) and observed (dashed bars) frequencies of TFs that interact with MDT-15 and that have a NHR-type DNA-binding domain, confer an altered Nile Red staining phenotype, or occur in the metabolic GRN (see Supplementary Table S5). **(C)** Images showing GFP expression in *Pacdh-1::GFP* (left) and *Pacdh-2::GFP* (right) transgenic animals that were subjected to control RNAi (top), *nhr-10* RNAi (middle) and *mdt-15* RNAi (bottom). Remaining GFP expression in *Pacdh-2::GFP* animals occurs in body wall muscle. Insets in the top right corners correspond to the DIC image of the animal. **(D)** Bar graphs representing quantifications of intestinal GFP expression shown in (C). Error bars indicate the s.e.m.

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.



**Figure 5** An integrated NHR network. Interactions involving NHRs that are in module II (right, beige background) and module III (left, green background), their targets and MDT-15 are represented. NHRs that specifically bind a single target gene that is part of either module were also included. Circles—NHRs; numbers depict NHR identity, that is ‘1’ is NHR-1; NR—Nile Red staining; ORO—Oil-Red-O staining.

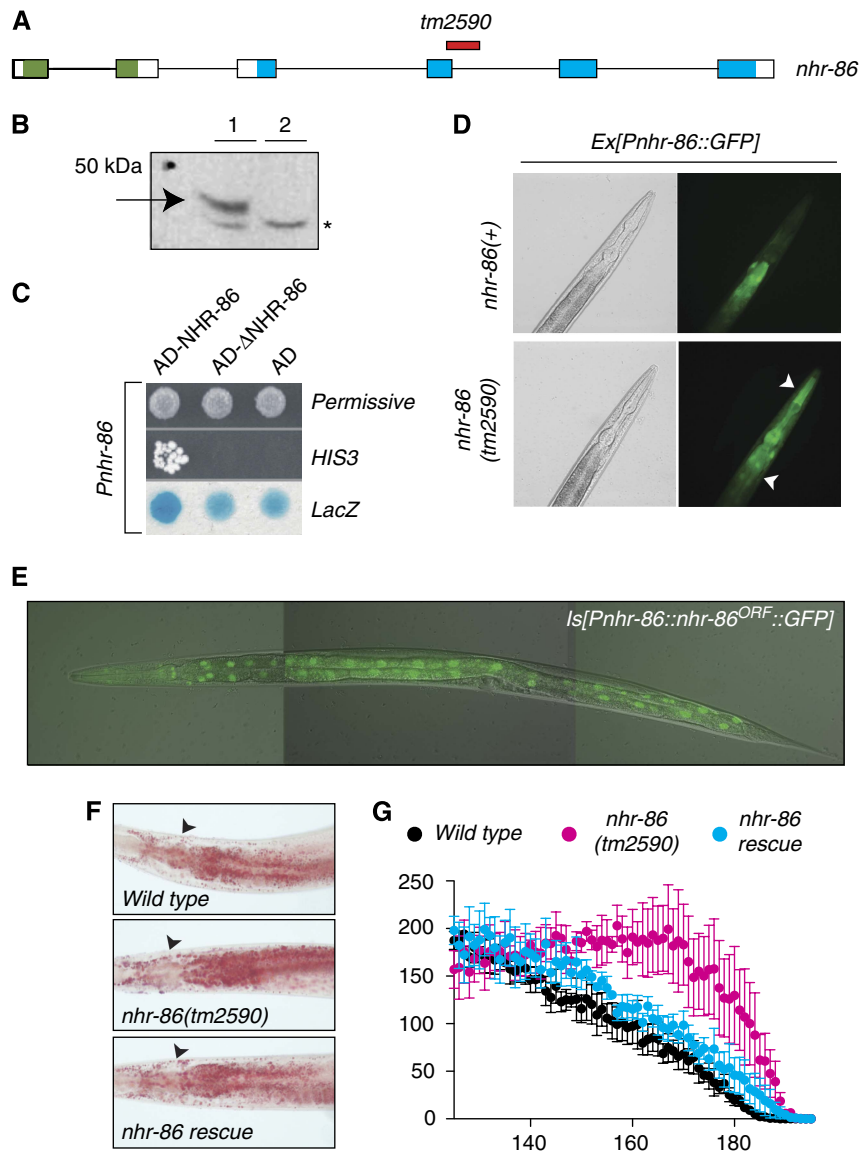
### NHR-178 and NHR-45 respond to nutrient availability

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) ( $100 \pm 0\%$  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 ( $84 \pm 1\%$  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 a *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 ( $100 \pm 0\%$ ) (Figure 7A). Microinjection of a wild-type *nhr-45* construct restored the intestinal and pharyngeal GFP expression ( $80 \pm 1\%$  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 ( $3.5 \pm 0.7\%$ ) *Pnhr-178::GFP* 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 ( $90 \pm 0.3\%$  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



**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<sup>ORF</sup>::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).

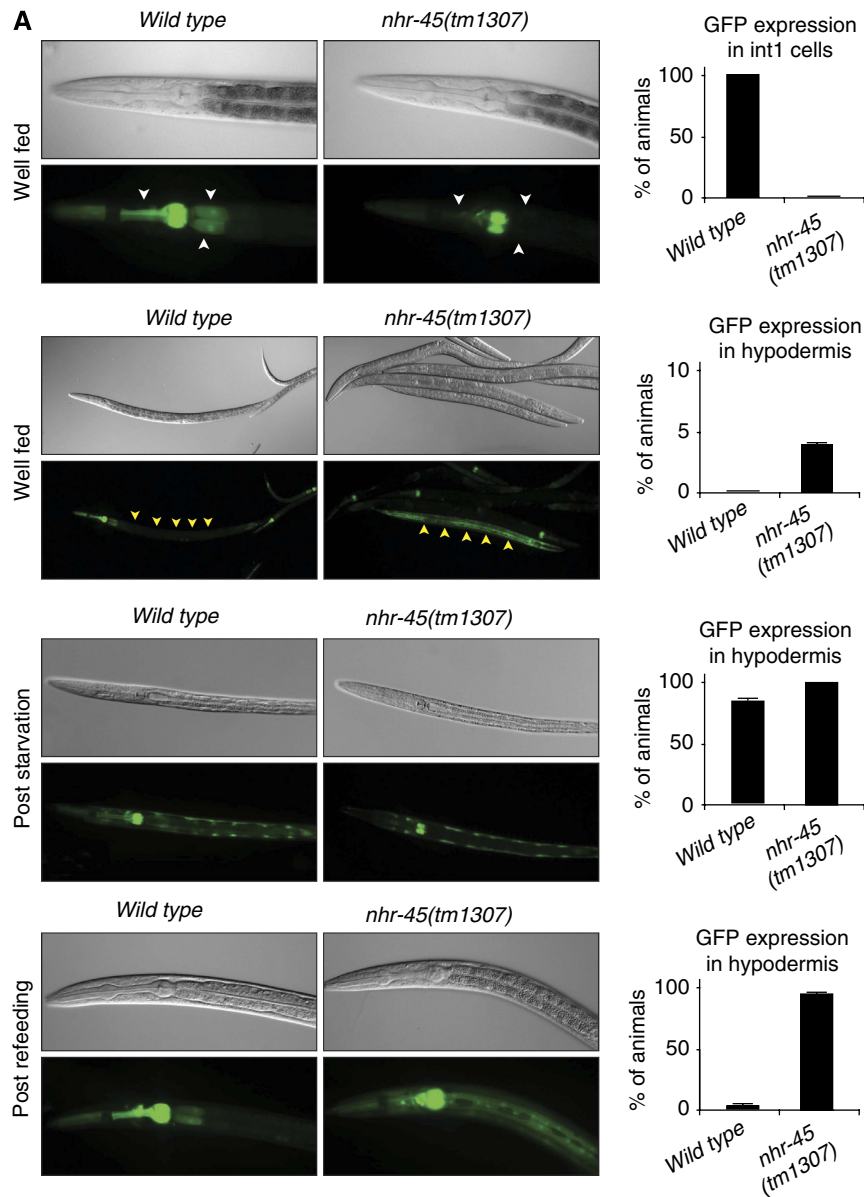
novel insights into the design principles of network organization and how this relates to network functionality. Altogether, our observations suggest that *C. elegans* physiological GRNs evolved to enable rapid and robust responses to environmental or physiological cues by a concurrence of NHR family 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

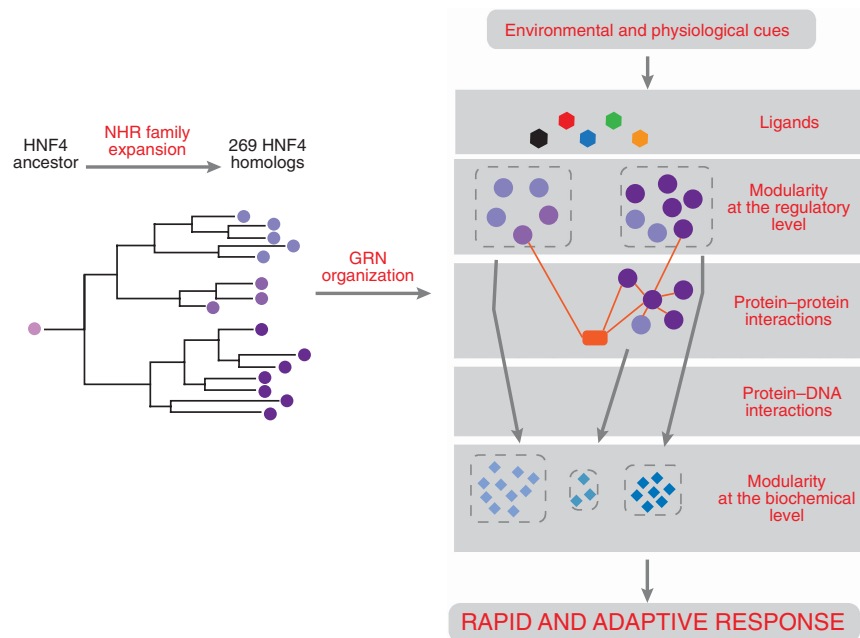




**Figure 7** An NHR gene circuit that responds to nutrient availability. **(A)** Images showing *Pnhr-178* activity in wild-type and *nhr-45(tm1307)* animals under different feeding conditions. Graphs next to each group of images show the percentage of animals that exhibit GFP expression in the indicated 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.

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 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.

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 hypodermis (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

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*, 2000b) 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*, 2000a). 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  $\geq 5$  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 pelleted 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 ~30 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 (Ashrafi *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  $-20^{\circ}\text{C}$ . This stock solution was then diluted 1:500 in  $1 \times$  phosphate-buffered saline, and overlaid on top of NGM plates either seeded with OP50 or with RNAi bacteria (HT115(DE3)). After the plates were equilibrated overnight at  $22^{\circ}\text{C}$ , synchronized L1 stage animals were placed onto these plates. When animals reached the L4 stage ( $\sim 36$  h later at  $20^{\circ}\text{C}$ ), the phenotype assessment was done by fluorescence microscopy (see below for details). To quantify Nile Red 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, followed by Dunnett's Multiple Comparison post test, using GraphPad Prism v.5.00 software.

## 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 ImageJ 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 Axiovision (Zeiss) software. GFP fluorescence images were obtained using a FITC filter (excitation 460–500 nm, emission 510–565 nm). Nile Red fluorescence images were taken using a rhodamine filter (excitation 525–555 nm, emission 575–630 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-86<sup>ORF</sup>::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 VL505 strain, which carries the *Pnhr-86::nhr-86<sup>ORF</sup>::GFP* construct (Supplementary Table S6). The *nhr-45* rescuing

fragment was PCR amplified using the forward primer CTCTTCATTATGCATTTTTGTTC and the reverse primer TCACTGGAACGTGAGAGTCA 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 487 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/ $\mu\text{l}$  of the purified PCR product along with 80 ng/ $\mu\text{l}$  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 YEPD liquid medium at  $30^{\circ}\text{C}$  with a starting density of OD600  $\sim 0.1$  until they reach an OD600  $\sim 0.6$ – $0.8$ . 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  $\mu\text{l}$  of  $2 \times$  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  $4^{\circ}\text{C}$  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 (SQFRPEKKEKSTCSIC, 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](http://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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