Integration of Metabolic and Gene Regulatory Networks Modulates the C. elegans Dietary Response

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SUMMARY

Expression profiles are tailored according to dietary input. However, the networks that control dietary responses remain largely uncharacterized. Here, we combine forward and reverse genetic screens to delineate a network of 184 genes that affect the C. elegans dietary response to Comamonas DA1877 bacteria. We find that perturbation of a mitochondrial network composed of enzymes involved in amino acid metabolism and the TCA cycle affects the dietary response. In humans, mutations in the corresponding genes cause inborn diseases of amino acid metabolism, most of which are treated by dietary intervention. We identify several transcription factors (TFs) that mediate the changes in gene expression upon metabolic network perturbations. Altogether, our findings unveil a transcriptional response system that is poised to sense dietary cues and metabolic imbalances, illustrating extensive communication between metabolic networks in the mitochondria and gene regulatory networks in the nucleus.

INTRODUCTION

To maintain homeostasis, a cell must be able to sense its own energy state, assess nutrient availability, and modulate metabolic pathways in a coordinated fashion. Organisms utilize and integrate endocrine, allosteric, and transcriptional mechanisms to respond to nutrients and activate or repress appropriate metabolic pathways accordingly. Different diets provide nutrients in different proportions and affect the transcription of metabolic genes in different ways. Dramatic changes in gene expression occur following dietary shifts in C. elegans, D. melanogaster, and M. musculus (Carsten et al., 2005; Du et al., 2010; MacNeil et al., 2013 [this issue of Cell]).

Ensuring optimal metabolic tuning to complex dietary signals requires integration of nutrient sensors and their downstream targets to produce a coherent response. Studies in yeast have revealed coexpression of metabolic enzymes as a central mechanism of nutrient response. For example, the yeast transcription factor (TF) Gcn4 activates enzymes involved in amino acid synthesis upon amino acid starvation (Hinnebusch, 2005). This type of coordinated, need-based metabolic transcriptional response to nutrients is an important mechanism in yeast, but the extent to which it occurs in more complex multicellular organisms remains largely unknown.

Inborn errors of metabolism are relatively rare recessive disorders that are characterized by a buildup of metabolites that cannot be processed and/or a lack of metabolites required for basic cellular processes (Saudubray et al., 2006). Patients with such diseases can display a variety of symptoms such as failure to thrive, developmental delay, and seizures (Acosta, 2010). These diseases are often clinically managed by altering the patient’s diet to avoid buildup of toxic metabolites and to supplement limiting nutrients. For example, maple syrup urine disease, a disorder in which patients fail to catabolize the branched chain amino acids (BCAAs) leucine, isoleucine, or valine, is managed by limiting the intake of protein-rich foods.

The nematode C. elegans is a free-living bacterivore that is a genetically tractable model instrumental in understanding mechanisms relating to human disease. For instance, genomescalar RNA interference (RNAi) resources have been used to identify essential genes (Kamath et al., 2003; Sönichsen et al., 2005), genes affecting fat storage (Ashrafi et al., 2003), and other processes. Further, the animal is transparent, which has enabled the use of green fluorescent protein (GFP) to monitor spatiotemporal gene expression in living animals (Chalfie et al., 1994; Grove et al., 2009; Hunt-Newbury et al., 2007; Martinez et al., 2008). The core metabolic networks and major nutrient-sensing pathways, including insulin and TOR, are highly conserved between C. elegans and humans. However, the TF family of nuclear hormone receptors (NHRs) that regulate metabolic gene expression has greatly expanded, with a total of 271 members, compared to 48 in humans (Reece-Hoyes et al., 2008).
C. elegans exhibits an altered gene expression profile when fed a bacterial diet of Comamonas DA1877 compared to the standard laboratory diet of E. coli OP50, and these changes are accompanied by altered life-history traits such as developmental rate, number of offspring, and lifespan (MacNeil et al., 2013). The metabolic gene acdh-1 exhibits the most dramatic change in expression between these diets. We developed a “dietary sensor” strain comprising the acdh-1 promoter driving the expression of GFP—when fed the standard laboratory diet of E. coli OP50, GFP expression is high, but on a Comamonas DA1877 diet, GFP expression is dramatically reduced (MacNeil et al., 2013).

Here, we perform forward and reverse genetic screens to identify genes that can alter the transcriptional response to diet. We identify 184 genes, including 146 “activators” and 38 “repressors.” Most of the 38 repressors encode enzymes that function within four mitochondrial metabolic pathways: BCAA breakdown, methionine metabolism, glycine cleavage system, and the tricarboxylic acid cycle (TCA). Human orthologs of most of the dietary response repressors confer inborn diseases of amino acid metabolism when mutated. In addition to acdh-1, the transcription of several other metabolic genes from these four pathways is modulated in response to both diet and metabolic network perturbations. These genes represent two modules that exhibit reciprocal behavior—one module, which includes acdh-1, is repressed by Comamonas DA1877 but is activated by specific metabolic network perturbations, whereas the other is activated by Comamonas DA1877 but is repressed by these metabolic network perturbations. We identify the TFs SBP-1, NRH-10, and NRH-68 as candidate mediators of the response to metabolic perturbations. Taken together, we uncover extensive communication between mitochondrial metabolic networks and nuclear gene regulatory networks, which may serve to optimize metabolic flux under different dietary and/or metabolic conditions.

RESULTS

A Forward Genetic Screen for Dietary Sensor Repressors

Animals harboring the Pacdh-1::GFP dietary sensor display much lower levels of GFP expression when fed Comamonas DA1877 than when fed the standard laboratory diet of E. coli OP50 (MacNeil et al., 2013). ACDH-1 has been annotated as an acyl-CoA dehydrogenase involved in β-oxidation of short chain fatty acids (Van Gilst et al., 2005) and as a branched chain acyl-CoA dehydrogenase involved in BCAA breakdown (Murphy et al., 2003). Sequence comparison of the ACDH-1 protein with the human ACADSB enzyme (which functions in BCAA breakdown) and the human SCAD enzyme (which functions in β-oxidation) indicates that ACDH-1 more closely resembles ACADSB (Figure S1 available online).

We performed a forward genetic screen to identify genes that, when mutated, cause activation of the acdh-1 promoter on a Comamonas DA1877 diet. We mutagenized the dietary sensor strain, screened ~10,000 genomes, and isolated 45 fertile F2 mutants that produced GFP-positive offspring (Figure 1A). These mutants varied in both level and pattern of GFP expression. For example, some mutants displayed relatively broad expression in the intestine, hypodermis, and muscle, whereas others express GFP only in the intestine (Figure 1B and Table S1). We mapped 20 mutations to four chromosomes. Five mutants were selected for whole-genome sequencing, one from each linkage group and one additional mutant from linkage group IV. We identified five mutations in four genes: mmcm-1, pccb-1, F32B6.2, and F25B4.1, all of which encode mitochondrial metabolic enzymes—mmcm-1 encodes methylmalonyl-CoA mutase, pccb-1 encodes the β subunit of propionyl-CoA carboxylase, and F32B6.2 encodes an ortholog of α methylcrotonoyl-CoA carboxylase. These three proteins are all involved in BCAA breakdown. The fourth gene, F25B4.1, encodes the T protein of the glycine cleavage system. We have named F32B6.2 “mccc-1” and F25B4.1 “gcst-1” to reflect these annotated biochemical functions. By complementation tests and sequencing, we identified additional alleles of three of the four genes (Figure 1C).

To verify that the mutations truly affect the expression of acdh-1, we examined endogenous messenger RNA (mRNA) levels in four mutants. When grown on Comamonas DA1877, all mutants showed an increase in acdh-1 mRNA levels relative to wild-type animals, which confirms the results obtained with the dietary sensor (Figure 1D, orange bars). We found similar changes in expression of two other diet-responsive genes, acdh-2 and ech-6, in these mutants (Figure 1D and data not shown). When fed E. coli OP50, the expression of these genes was similar in wild-type and mutant animals (Figure 1D, purple bars), demonstrating that the mutations do not cause a general increase in their expression. In three of the four mutants, the expression of acdh-1 was lower on Comamonas DA1877 than on E. coli OP50, indicating that they are still somewhat able to respond to dietary cues (Figure 1D, compare orange and purple bars). The dietary response was completely impaired only in the mmcm-1(ww5) mutant (Figure 1D).

The acdh-1 promoter not only responds to different bacterial diets but is also repressed upon starvation (MacNeil et al., 2013; Van Gilst et al., 2005). We tested the response to starvation in our mutants. When starved for 24 hr, mccc-1(ww4) mutant animals retain GFP expression, which indicates that the starvation response is retained in these mutants as well. In contrast, in mmcm-1, pccb-1, and gcst-1 mutants, GFP expression was reduced, indicating that the response to starvation is retained (Figure 1E). mmcm-1(ww5) mutants are completely impaired in the dietary response (Figure 1D) but retain a starvation response, confirming that these two responses are distinct (MacNeil et al., 2013). Altogether, these observations show that the dietary sensor can respond not only to diet but also to endogenous metabolic network perturbations.

A Genome-Scale RNAI Screen

To identify additional genes that can affect the dietary sensor, we explored the use of RNAi by feeding, which is carried out in E. coli HT115 bacteria (Timmons et al., 2001). When fed E. coli HT115, the Pacdh-1::GFP animals express intermediate levels of GFP,
**Figure 1. Forward Genetic Screen for Dietary Sensor Regulators**

(A) Flow chart of EMS mutagenesis screen.
(B) Examples of GFP expression in wild-type animals and two mutant strains.
(C) Cartoon illustrating amino acid changes in four proteins identified in the forward genetic screen. BD, biotin binding domain; B12B, vitamin B12 binding domain.
(D) Endogenous acdh-1 and acdh-2 levels in mutants as measured by nCounter assays and normalized to ama-1.
(E) Mutants exhibit different responses to starvation.

See also Tables S1 and S2.
enabling us to identify both dietary sensor repressors and activators. In addition, we took advantage of the fact that *Comamonas* DA1877 can be diluted and still repress the dietary sensor (MacNeil et al., 2013). Adding a small amount of *Comamonas* DA1877 to the *E. coli* HT115 RNAi feeding lawn repressed the dietary sensor while maintaining RNAi knockdown efficiency (Figure 2A). This enabled us to perform a genome-scale RNAi screen in the presence of *Comamonas* DA1877 (Figure 2B).

We screened the ORFeome RNAi library, which covers more than half of all predicted protein-coding genes (Rual et al., 2004). After screening the library once in each dietary condition, we obtained 836 hits (8%, Figure 2B). These hits were rearrayed and retested four times to remove false positives. The 554 genes that rescored in at least two of four retests were considered further. This list of hits contained many genes involved in general protein expression that may not specifically affect the dietary sensor. To identify such potential false positives, we screened the 554 hits against another transgene, *Pmir-63::GFP*, that also expresses GFP in the intestine but is not sensitive to *Comamonas* DA1877 (Figures 2A and 2B). We retained 179...
high-confidence hits (~2% of the genes tested). Of these, 146 caused a decrease in GFP expression (activators), whereas 33 caused an increase (repressors) (Table S2).

We retrieved nhr-10 and mct-15, known activators of acdh-1 (Arda et al., 2010), as well as two of the genes (mcc-1 and gcc-1) found in the forward genetic screen. The other two genes (mmcm-1 and pccb-1) were not retrieved, although they were present in the RNAi library. This could be due to the inherent variability and the relatively high false negative rate of RNAi experiments (Kamath et al., 2003). Indeed, when we retested these clones in a small-scale, directed RNAi experiment, we found that their knockdown does increase GFP expression (data not shown).

RNAI Screen Validation
To verify that the genes found by RNAi affect endogenous acdh-1 expression, we used quantitative RT-PCR (qRT-PCR) in wild-type (N2) animals subjected to RNAi of 11 representative genes found in the forward screen. As shown in Figure 2C, the qRT-PCR recapitulated what was observed in the Pacdh-1::GFP dietary sensor strain for all 11 knockdowns.

We obtained C. elegans mutant strains for several genes found in the RNAi screen, including the TFs nhr-68 and htl-11, and the enzymes ZK669.4, sams-5, sams-1, and metr-1 (Figure S2). We introduced each mutation into the Pacdh-1::GFP dietary sensor strain and examined GFP levels on E. coli OP50 and Comamonas DA1877 (Figure 2D). These mutants exhibited GFP expression levels that recapitulate the effects observed by RNAI—mutations in the enzymes and htl-11 all activated GFP expression, whereas a deletion in nhr-68 decreased GFP expression. Further, we confirmed the increased expression of endogenous acdh-1 in animals fed E. coli OP50 and Comamonas DA1877 by qRT-PCR in Δhtl-11 and Δsams-5 mutant animals (Figure 2E).

To probe potential functional relationships among the genes discovered in the genetic screens, we assessed their connectivity in WormNet, a probabilistic functional gene network constructed through integration of different data types (Lee et al., 2008). The combined 181 genes found by both the forward and reverse genetic screens are significantly connected in WormNet and form a closely linked functional network (p = 1.97 × 10−78) (Figure S3). Thus, these 181 genes are more functionally interrelated than would be expected for a random set of genes, supporting the overall quality of the RNAi screen.

Dietary Sensor Activators
The majority (74%) of the 146 activators are expressed in the intestine (Figure S3), suggesting that they may act cell autonomously. Gene ontology (GO) analysis revealed an enrichment for several terms, including “growth,” “sex differentiation,” and “ribosomal biogenesis” (Figure S3). Several of these genes are predicted to function in a number of biological processes, including general gene expression, splicing and translation, metabolism, and the regulation of transcription (Figure S3). Because acdh-1 is also repressed in response to starvation, the dietary sensor strain cannot discriminate between genes involved in, or inducing, a starvation response and those involved in the dietary response. To identify which activators may mediate a starvation response and which may be more specific to the dietary response, we used two additional transgenic strains in which GFP expression is repressed by starvation, but not affected by bacterial diet (Pgst-4::GFP and PCS3A3.2::GFP, Figure S4). We found 37 activators that affect at least one of these transgenes, 12 of which affected both, indicating that they may induce or mediate a starvation rather than dietary response (Table S3). The remaining 109 activators were specific to Pacdh-1::GFP.

Dietary Sensor Repressors Function in Four Metabolic Pathways
The combined 35 dietary sensor repressors obtained in both screens are enriched for GO terms relating to metabolism, including “mitochondrion,” “TCA cycle,” and “acetyl-CoA catabolic process” (Figure S3). Overall, 83% of these are annotated metabolic genes, compared to 15% of the activators, and the vast majority are expressed in the intestine (Figure S3). Interestingly, most (24) of the repressors encode enzymes specifically involved in four metabolic pathways: BCAA breakdown (n = 8), methionine metabolism (n = 5), the glycine cleavage system (n = 2), and the TCA cycle (n = 9) (Figure 3).

We wondered whether other genes in these pathways that were not retrieved in the genetic screens would affect the dietary sensor as well. We obtained deletion mutants for two genes involved in BCAA breakdown (mce-1 and pcca-1) and one from the methionine metabolism pathway cbl-1 (Figure 3). These deletions were introduced into the Pacdh-1::GFP dietary sensor strain. Deletions in mce-1 and pcca-1 both caused increased GFP expression on all three diets, and a deletion in cbl-1 caused increased GFP on Comamonas DA1877, but not on E. coli OP50 (Figure S5). Altogether, these findings indicate that disrupting these four metabolic pathways affects the dietary response to Comamonas DA1877. The observation that loss of several individual genes within a common pathway can induce similar changes in the expression of acdh-1 suggests that these perturbations may converge onto a common regulatory signal.

Interestingly, acdh-1 itself was found as a repressor in the RNAI screen (Table S2). We obtained an acdh-1 deletion mutant and introduced it into the Pacdh-1::GFP dietary sensor strain. We observed higher GFP levels in the acdh-1 mutant relative to the wild-type dietary sensor strain when the animals were fed E. Coli OP50 or HT115 diets but observed less of an increase on a Comamonas DA1877 diet. On the latter diet, acdh-1 expression is normally low (Figure 4A). Thus, feedback on the acdh-1 promoter occurs mainly on diets in which endogenous acdh-1 levels are normally high, suggesting that acdh-1 expression is regulated according to need.

Cellular metabolic needs are unlikely to be met by modulating the expression of a single enzyme; rather, enzymes within a metabolic pathway or network may need to be coordinately regulated (Grünig et al., 2010). In addition to acdh-1, several other genes predicted to function within BCAA breakdown and methionine metabolism pathways are also transcriptionally regulated in response to diet (Figure 3) (MacNeil et al., 2013). This suggests that these genes comprise a coordinately regulated module. We wondered whether these genes were also transcriptionally sensitive to metabolic network perturbation. We examined the expression of seven Comamonas
DA1877-responsive genes (four from BCAA breakdown, two from methionine metabolism, and one NHR) by qRT-PCR in several mutant strains that exhibit altered acdh-1 expression (Dmetr-1, Dsams-1, Dme-1, Dacdh-1, and Dhh-11). We found that several BCAA breakdown genes, as well as nhr-68, are coinduced with acdh-1 in response to metabolic network perturbation (Figure 4B). This further supports the modular regulation of these functionally related genes and indicates that this may perhaps be mediated by nhr-68 (see below). The expression of acdh-2, a close homolog of acdh-1, is also increased in an acdh-1 deletion mutant, which may indicate a compensation mechanism of acdh-2 for the loss of acdh-1 function. Interestingly, the Comamonas DA1877-induced gene cbs-1 (MacNeil et al., 2013) exhibited opposite behavior compared to the Comamonas DA1877-repressed genes because it is repressed in the metabolic gene mutants.

To characterize the transcriptomic response to metabolic gene perturbations in more detail, we performed a microarray expression profiling experiment with two metabolic gene mutants on a Comamonas DA1877 diet. One of these genes, pcca-1, functions in BCAA breakdown, whereas the other, metr-1, is involved in methionine metabolism. The two mutations elicit highly similar changes in gene expression (Figure 4C), further suggesting that these two different metabolic mutations may cause similar metabolomic changes that may impinge on a common regulatory signal. Strikingly, most of the core genes that change in response to a Comamonas DA1877 diet (MacNeil et al., 2013) also exhibited expression changes in one or both metabolic mutants (Figure 4D). In fact, there are two modules of coexpressed genes that exhibit reciprocal behavior—one that is repressed by a Comamonas DA1877 diet but is activated in the metabolic gene mutants and one that is activated by a Comamonas DA1877 diet but is repressed in the metabolic gene mutants (Figure 4E and Table S4).

**Human Orthologs of Dietary Repressors Are Involved in Inborn Metabolic Disease**

Many metabolic reactions are evolutionarily conserved, and most of the C. elegans enzymes found in our genetic screens have human orthologs (Table S2). Most of these are associated with human inborn metabolic diseases. For example, human orthologs of the dietary repressors, such as pcca-1 and metr-1, are found in several human inborn metabolic diseases, including phenylketonuria, homocystinuria, and maple syrup urine disease. These findings suggest that the evolutionarily conserved metabolic pathways in C. elegans may provide insights into the underlying mechanisms of human inborn metabolic diseases.
with inborn disorders of amino acid metabolism (Figure 5). For instance, we identified multiple orthologs of genes that, when mutated in humans, cause maple syrup urine disease, methylmalonic acidemia, homocystinuria, or propionic acidemia. These diseases are characterized by toxic buildups of amino acids and intermediate metabolites and are treated by limiting amino acid (protein) intake and supplementing vitamins and other cofactors (Figure 5). We also found orthologs of TCA cycle genes that cause diseases such as lactic acidosis and pyruvate dehydrogenase deficiency. These may also result in the accumulation of intermediate metabolites or in the inability to generate sufficient amounts of energy (ATP).

Figure 4. Metabolic Feedback and Transcriptional Compensation
(A) Increased acdh-1 promoter activity in Δacdh-1 mutants identifies feedback control.
(B) qRT-PCR of seven genes (rows) in different strains (columns).
(C) Overlap in gene expression changes between two metabolic gene mutants.
(D) Overlap between genes that change in expression in the two metabolic gene mutants and those that change in response to a Comamonas DA1877 diet.
(E) Opposite changes in gene expression in response to a Comamonas DA1877 diet versus metabolic network perturbations.

See also Figure S2 and Table S4.
Figure 5. Orthologs of Dietary Sensor Repressors Confer Human Inborn Metabolic Disorders Relating to Amino Acid Metabolism

Network connecting dietary sensor repressors, C. elegans phenotypes, human orthologs, human inborn metabolic diseases, and dietary treatments. Orange nodes, C. elegans phenotypes; colored squares, C. elegans genes found in the screen; diamonds, human orthologs/homologs; gray circles, human diseases; hexagons, nutrients. Red edges indicate dietary avoidance; green edges indicate dietary supplementation.
Mutations in two C. elegans metabolic genes (\(\Delta m\)etr-1 and \(\Delta pcc\)ca-1) cause highly similar changes in gene expression (Figure 4C). \(m\)etr-1 is the ortholog of human methionine synthase (MTR), which, when mutated, causes methylcobalamin (vitamin B12) deficiency. \(pcc\)ca-1 is the ortholog of human PCCA, which, when mutated, causes propionic acidemia. Our observations suggest that mutations in these genes may result in similar metabolic effects. The human disorders caused by mutations in these genes are treated in part by supplementation with vitamin B12, and both disorders can be revealed by elevated propionylcarnitine levels in newborn screening (Weisfeld-Adams et al., 2010). This suggests that, as in C. elegans, these two human diseases have at least partially overlapping molecular phenotypes.

**Limited Involvement of TOR and Insulin Signaling Pathways in Response to Metabolic Network Perturbations**

To determine whether known nutrient sensing pathways such as TOR or the insulin signaling pathway are involved in upregulating acdh-1 in response to metabolic network perturbations, we performed RNAi on a panel of genes from these pathways in metabolic mutants harboring the Pacdh-1::GFP transgene. Knockdown of daf-2 or daf-16 had no effect on GFP expression in any of the mutants (Figure 6A). We also crossed the \(\Delta m\)etr-1 and \(\Delta acdh-1\) metabolic mutations into a transgenic strain expressing a DAF-16::GFP fusion protein to determine whether these metabolic mutants affected DAF-16 nuclear localization and found that neither had any effect (data not shown). Thus, insulin signaling does not mediate the response to metabolic network perturbations.

The TOR pathway is an attractive candidate for mediating the effects of metabolic perturbations because it affects acdh-1 in wild-type animals and is regulated by amino acid levels, specifically leucine (Laplante and Sabatini, 2012). We tested whether the TOR pathway is involved in mediating the response to endogenous metabolic network perturbations and found limited involvement (Figures 6A and 6B). Knockdown of rheb-1 or ruvb-1 reduced GFP levels in most metabolic gene mutants. However, in almost every case, GFP was still higher in these mutant strains than in the wild-type strain (Figures 6A and 6B). Therefore, it is likely that TOR signaling may be partially involved in, but not solely responsible for, mediating the induction of acdh-1 in metabolic mutants.

**Multiple TFs Affect the Response to Metabolic Network Perturbations**

In wild-type animals, both nhr-10 and nhr-23 activate the dietary sensor (Arda et al., 2010; MacNeil et al., 2013). In the RNAi screen, we identified additional TFs that affect acdh-1 expression, most of which are NHRs (Table S2). We knocked down nhr-10, nhr-23, nhr-68, nhr-173, and nhr-74, as well as sbp-1, by RNAi in multiple metabolic gene mutants and examined changes in GFP expression. We observed complex gene regulatory effects of different TFs (Figures 6A and 6B). First, sbp-1 RNAi resulted in a dramatic decrease in GFP expression in all animals tested, indicating that SBP-1 might function at the top of the metabolic gene regulatory hierarchy. However, sbp-1 RNAi also reduced GFP expression in the two starvation-responsive transgenic strains (Table S3). Previously, SBP-1 has been shown to regulate genes involved methionine metabolism (Walker et al., 2011). Altogether, these observations suggest that SBP-1 may be a general regulator of metabolic gene expression in the C. elegans intestine. Second, nhr-23 RNAi has only mild effects on GFP expression in most mutants, whereas it has stronger effects in the wild-type sensor strain, suggesting that it primarily controls acdh-1 expression in response to diet. However, it is important to note that nhr-23 RNAi was performed under dilute conditions to avoid larval arrest. Third, nhr-10 and nhr-68 knockdown dramatically reduces GFP expression in almost all mutants. For example, GFP levels in mce-1 and pcca-1 deletion animals are equivalent to those in wild-type animals subjected to nhr-10 or nhr-68 knockdown (Figure 6B). Therefore, nhr-10 and nhr-68 may mediate the increase in acdh-1 expression in response to specific metabolic network perturbations.

Knockdown of nhr-10 in an nhr-68 deletion mutant or vice versa had little additional effect on GFP expression, indicating that these two NHRs may function together (Figure 6A). Knockdown of the other NHRs tested, nhr-173 and nhr-74, had mild effects on GFP expression in wild-type animals and had similarly mild effects in the context of metabolic network perturbations. However, knockdown of nhr-173 further reduces GFP expression in an nhr-10 deletion mutant, indicating that these NHRs may function in parallel.

**DISCUSSION**

We have unraveled a complex network of genes that affect dietary gene expression in C. elegans. This network is likely not yet complete for several reasons. First, forward genetics identified mutations in four metabolic genes, and although we have multiple alleles for three of these genes, the screen is not yet saturated. Second, the reverse genetic RNAi screen has not revealed all genes involved because the ORFeome RNAi library contains only half of all C. elegans protein-coding genes (Rual et al., 2004) and because RNAi screens are prone to a high false negative rate (Kamath et al., 2003). This is supported by the initial retrieval of only two of the four genes found by forward genetics and the identification of additional genes through targeted analysis of mutants in the metabolic pathways involved.

We primarily focused on dietary sensor repressors, most of which encode mitochondrial enzymes. We found that these repressors function in an intricate feedback and compensation system that results in regulation of metabolic gene expression. This is perhaps best exemplified by the feedback in which deletion of acdh-1 results in an increase in its own promoter activity under dietary conditions when acdh-1 levels are normally high. We know that this feedback extends from acdh-1 to other genes involved in BCAA breakdown because additional genes in this pathway either change in expression in response to a Comamonas DA1877 diet, confer an increase in acdh-1 promoter activity when perturbed, or both. Thus, as has been observed in simpler unicellular organisms such as bacteria and yeast (Grüning et al., 2010), metabolic changes induced genetically or by diet likely result in disruption of metabolic flux that, in turn, elicit a compensatory transcriptional response of relevant metabolic genes.
Altogether, our study reveals extensive communication between mitochondrial metabolic networks and nuclear gene regulatory networks that function to dial metabolic gene expression according to cellular or organismal need (Figure 7).

The mechanisms by which information is relayed from the mitochondria to the nucleus remain poorly understood. There are several shared metabolites among the four metabolic pathways found in the screens, including acetyl-CoA, succinyl-CoA, pyruvate, and the cofactor cobalamin (vitamin B12) (Figure 3). These shared metabolites represent points of convergence for metabolic flux through each of the individual pathways. Perturbing flux through methionine metabolism by mutating metr-1, for instance, may affect flux through BCAA breakdown due to buildup or depletion of shared metabolites. This could explain the observation that several genes from these two pathways are coregulated in response to diet and metabolic perturbation in either pathway. These metabolites may mediate the communication from the mitochondria to the nucleus. It is well known that these metabolites have broad ranging effects on the cell. For instance, epigenetic effects can be mediated...
by histone acetylation, methylation, and phosphorylation, which require acetyl-CoA, SAM, and ATP, respectively. It is possible that overall or specific protein modification levels are affected by metabolic network perturbations and result in changes in gene expression. Further, these metabolites may directly or indirectly target TFs such as NHRs (see below).

Interestingly, there was a high degree of overlap in both upregulated and downregulated genes in the gene expression profiles of two metabolic gene mutants, \( \Delta \text{metr-1} \) and \( \Delta \text{pcca-1} \). This suggests that the functional defects caused by perturbing either the BCAA breakdown pathway or methionine metabolism may elicit similar metabolic shifts in the animal, which may impinge upon a common regulatory signal. Both metabolic gene mutations reverse many of the gene expression changes conferred by a \( \text{Comamonas DA1877} \) diet in wild-type animals.

Two modules of coexpressed genes exhibit reciprocal “see-saw” behavior—one module is repressed by a \( \text{Comamonas DA1877} \) diet but is activated in the metabolic mutants, whereas another is activated by a \( \text{Comamonas DA1877} \) diet but is repressed in the metabolic gene mutants. This implies that both metabolic network perturbations and a \( \text{Comamonas DA1877} \) diet converge onto the same regulatory network to elicit converse effects on the two modules, perhaps via two opposing regulatory signals (Figure 7).

NHR-10 directly binds the \( \text{acdh-1} \) promoter (Arda et al., 2010) and mediates the induction of \( \text{acdh-1} \) in response to metabolic network perturbations in several mutants, particularly those that function within BCAA breakdown. In addition, NHR-68 and several other NHRs either function together or in parallel with NHR-10. Epistasis experiments with additional NHRs, as well as other TFs, will further illuminate their function in metabolic gene regulation. NHRs are ligand-regulated TFs that may be able to directly sense metabolite accumulation and regulate genes accordingly. For instance, NHR ligands may be produced as a result of specific metabolic network perturbations. Alternatively, NHRs may be inactivated by a metabolite, and depletion

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Figure 7. Model for Integration of Metabolic Network Perturbation and Dietary Response

Perturbing metabolic networks in mitochondria may lead to imbalances that are sensed by gene regulatory networks in the nucleus and may interfere with the dietary response.
of that metabolite upon network perturbation might result in NHR activation. Perturbation of different metabolic pathways may result in the accumulation or depletion of metabolites that may be sensed by different NRHs with overlapping downstream targets. Previously, we found functional modularity of NRHs in a C. elegans gene regulatory network (Arda et al., 2010). Here, we find a modular organization at the level of metabolic gene expression. Together, these findings support a model in which gene regulatory network modules facilitate responses to physiological and environmental cues.

Most dietary sensor repressors encode enzymes involved in inborn disorders of amino acid metabolism in humans. These diseases are usually treated by dietary interventions that are designed to (1) avoid buildup of toxic metabolites, (2) supplement patients with depleted metabolites, and/or (3) supplement cofactors that may improve residual enzyme activity (Figure 5). In C. elegans, mutations in genes from different pathways within the metabolic network (BCAA and methionine metabolism) can cause highly similar changes in gene expression. Thus, it may be that a dietary regimen used to mitigate one type of metabolic disease may also be beneficial for another. C. elegans presents an attractive model with which transcriptional changes in response to different diets and metabolic network perturbations can be compared and potentially can be used in screens for drugs or other small molecules that affect these changes.

Our study establishes C. elegans as a powerful model to dissect the mechanisms of dietary responses, inborn metabolic diseases, and the connections between them. It is likely that other parts of the metabolic network respond to different cues. We provide a framework to identify candidate sensors to these cues and to combine these sensors with genetic screens and epistasis to dissect the mechanisms involved.

**EXPERIMENTAL PROCEDURES**

**Strains**

C. elegans strains were grown as described (Brenner, 1974). N2 (Bristol) was used as the wild-type strain. The following mutant strains were provided by the C. elegans Gene Knockout Consortium (CGC): nhr-68(gk708), sams-1(ok2946), cbl-1(ok2954), mce-1(ok243), pcca-1(ok2282), metr-1(ok521), ZK669.4(ok3001), sams-5(gk147), and hth-11(ok2944). The nhr-10(tm695) mutant strain was provided by the National Bioresource Project, Japan. The Pacdh-1::GFP strain (VL749) is described in MacNeil et al. (2013). VL405 [Pmir-63::GFP + unc-119(+)] is described in Martinez et al. (2008). BC12350 dpy-5(e907); slt12119 [Cre;Cs35A3.2::GFP + Ceh361] is described in McKay et al. (2003). CL2166 dls19[pAF151(Pgst-4::GFP::NSL)] was obtained from the CGC (Tawe et al., 1998).

**EMS Screen**

VL749 animals were treated with 0.5% ethyl methyl sulfoxide (EMS) for 4 hr. Mutagenized animals were transferred to nematode growth media (NGM) plates seeded with Comamonas DA1877 and allowed to lay eggs. Eggs were collected, and 100 10 cm OP50, HT115, or E. coli OP50, HT115, or Comamonas DA1877 NGM plates were seeded with 50 F1 animals each. F2 animals were screened for visible GFP. A single GFP-positive F2 animal was picked from each F1 plate and transferred to individual plates. Of the 100 mutants, 45 were fertile and produced GFP-positive offspring when fed Comamonas DA1877.

**Mutant Mapping**

Chromosomal assignment was done by crossing mutants carrying the Pacdh-1::GFP reporter into the CB4856 (Hawaiian) strain followed by SNP mapping of GFP-positive pools of F2 animals as described (Wicks et al., 2001). Mutants that produced GFP-positive F1 males were assigned to linkage group (LG) X. Primers used for chromosomal assignment are as described (Davis et al., 2005). Following chromosomal assignment, fine mapping was undertaken for five mutants using chromosomal primers as described (Davis et al., 2005; Wicks et al., 2001). We created him-5(e1467) lines carrying the Pacdh-1::GFP transgene and the mutation of interest to perform complementation tests. These him-5 lines were used to generate males that were crossed into mutant hemaphrodites carrying Pacdh-1::GFP, and GFP expression was assessed in F1 animals grown on Comamonas DA1877.

**Whole-Genome Sequencing**

Before sequencing, mutants were outcrossed three times to N2 wild-type animals. Total genomic DNA was prepared for mutants ww2, ww4, ww10, ww11, and ww17 as described (Sarin et al., 2010). Libraries were made and barcoded using the NextFlex barcoding system and library construction kits (Bioo Scientific). Samples were sequenced by the IIGB Genomic Core facility, UC Riverside, using Illumina’s HiSeq2000 platform (51 bp reads). After filtering low-quality reads using the default Illumina pipeline quality filter, we recovered 184.8 million reads representing an 18× average coverage. Reads were mapped onto the C. elegans genome sequence (WS201), and variants were identified using MAQgene with default parameter setting except that minimum fraction of non-wild-type reads was set to 0.8, minimum span of uncovered bases to report as uncovered region was set to 1, and maximum sum of error qualities was set to 150 (Bigelow et al., 2009). Mismatches were compared between all five mutants. Base pair changes common to all mutants were ignored, as these were likely present in our starting strain. Variants were validated by PCR and sequencing. Additional alleles were identified by PCR amplification, followed by sequencing of targeted genes.

**RNAi Screen**

RNAi screening was performed on 96-well plates using NGM agar containing 5 mM IPTG. E. coli HT115 RNAi cultures were grown in LB containing 100 μg/ml ampicillin to log phase in 96-well deep well dishes. Bacteria were centrifuged and resuspended in 1/20 volume of M9. 10 μl of the resuspended cultures was added to the RNAi screening plates. For the Comamonas DA1877 condition screen, Comamonas DA1877 was grown overnight to saturation in Luria-Bertani (LB), then sonicated and diluted 1/10 with LB containing 100 μg/ml ampicillin. 10 μl drops were added to each well of the E. coli HT115-RNAi seeded plates. VL749 animals were synchronized by hypochlorite bleaching and washed in M9 media, and ~25 eggs were added to each well. After 60 hr, animals reached the early adult stage and were visually screened for GFP expression. RNAi clones found to affect GFP in the first pass of the screen (856 clones) were rearrayed and retested four times. 554 genes tested at least two out of four times and were rearrayed again. These clones were tested three times for specificity using strain VL405, which contains an integrated Pmir-63::GFP construct (Martinez et al., 2008). 192 genes did not affect Pmir-63::GFP in any of the three tests and were considered specific for Pacdh-1::GFP. All final hits were sequence verified.

**Mutant Validation**

nhr-68(gk708), sams-1(ok2946), cbl-1(ok2954), mce-1(ok243), pcca-1(ok2282), metr-1(ok521), ZK669.4(ok3001), sams-5(gk147), hth-11(ok2944), nhr-10(tm695), and hth-11(ok2944) mutants were outcrossed three times to the wild-type N2 strain and homozygosed with the exception of the ok3001 allele, a deletion in the gene ZK669.4, which produced sterile homozygotes. Pictures of sterile ZK669.4(ok3001) homozygotes from heterozygous parents were taken, and animals were retrospectively genotyped. RNA was isolated from the hth-11 and sams-5 deletion mutant strains grown on E. coli OP50, E. coli HT115, or Comamonas DA1877 for qRT-PCR analysis. All outcrossed mutant strains were also crossed to VL749 and homozygosed.

**qRT-PCR**

Animals were synchronized and grown on E. coli OP50, E. coli HT115, or Comamonas DA1837, and ~1,000 adult animals were harvested for each condition. Animals were thoroughly washed in M9 buffer, and total RNA was isolated using Trizol (Invitrogen), followed by DNase I treatment.
and cleanup using QIAGEN RNeasy columns. Complementary DNA (cDNA) was prepared from 1 μg of RNA using oligo-dT and Mu-MLV enzyme (NEB). Primer sequences for qRT-PCR were generated using the GETprime database (Gubelmann et al., 2011) and are listed in Table S5. qRT-PCR was performed in triplicate with the Applied Biosystems StepOnePlus Real-Time PCR System and Fast Sybr Green Master Mix (Invitrogen). Relative transcript abundance was determined using the ΔΔCt method and normalized to averaged ama-1 and act-1 mRNA expression levels (Livak and Schmittgen, 2001).

nCounter Analysis
RNA was extracted as described above. nCounter analysis was performed as per manufacturer’s instructions (NanoString Technologies) using 300 ng of total RNA, and expression between samples was normalized to ama-1 mRNA levels. Probes used are listed in Table S5.

Functional Annotation
GO analyses were performed using David (Huang et al., 2009). WormNet v.2 was used as described (Lee et al., 2008). Genes found to affect GO analyses were performed using David (Huang et al., 2009). WormNet v.2 functional annotation was used as described (Lee et al., 2008). Genes found to affect GO analyses were performed using David (Huang et al., 2009). WormNet v.2 was performed by the Genomics Core facility at University of Massachusetts Medical School using C. elegans genome arrays (Affymetrix). The RMA C. elegans genome sequence analysis. Nat. Genet. 421, 268–272. Benjami, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc., B 57, 289–300.


SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.02.050.

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REFERENCES


Figure S1. Cartoon of Homology with ACDH-1 Protein, Related to Figure 3
Cartoon depicting the ACDH-1 protein with percent sequence identity with human and C. elegans enzymes in the acyl-CoA dehydrogenase N-terminal (N), middle (M) and C-terminal (C) domains indicated.
Figure S2. Cartoon of Deletion Mutants, Related to Figure 4
Cartoons depicting gene structures of deletion mutants, with the exception of the mce-1 mutant for which the deletion has not been precisely mapped. Arrows indicate direction of transcription. Proteins are diagrammed to the right of the gene structures with N and C termini relative to depicted gene directionality. Color blocks on genes correspond to colored protein domains, which were determined using PFAM. Red lines on protein cartoons indicate the region affected by the deletion.
Figure S3. Functional Annotation of Dietary Sensor Regulators, Related to Figure 2

(A) Left – network indicating predicted functional connections between dietary sensor regulators in WormNet. Blue indicates dietary sensor activators and yellow indicates dietary sensor repressors. Right – receiver operator curve illustrating significance of connections shown on left. The dashed line on the diagonal indicates random associations.

(B) Significantly enriched GO terms for dietary sensor activators.

(C) Tissue expression and functional category distribution of dietary sensor activators, with and without “unknown” (in gray).

(D) Significantly enriched GO terms for dietary sensor repressors.

(E) Tissue expression and functional category distribution for dietary sensor repressors, with and without “unknown” (in gray).
Two additional transgenic reporters, *Pgst-4::GFP* and *PC53A3.2* exhibit decreased GFP expression in response to starvation.

*Figure S4. Two Transgenic Reporters that Respond to Starvation, Related to Figure 2*

Two additional transgenic reporters, *Pgst-4::GFP* and *PC53A3.2* exhibit decreased GFP expression in response to starvation.
Figure S5. Additional Mutants in BCAA Breakdown and Methionine Metabolism Pathways, Related to Figure 3

Differential interference contrast (DIC) and fluorescent images of wild-type animals compared to mce-1, pcca-1 and cbl-1 mutant animals harboring the dietary sensor, grown on either E. Coli OP50, E. Coli HT115 or Comamonas DA1877.

<table>
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<th>Wild Type</th>
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