A Gene-Centered *C. elegans* Protein-DNA Interaction Network

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SUMMARY

Transcription regulatory networks consist of physical and functional interactions between transcription factors (TFs) and their target genes. The systematic mapping of TF-target gene interactions has been pioneered in unicellular systems, using "TF-centered" methods (e.g., chromatin immunoprecipitation). However, metazoan systems are less amenable to such methods. Here, we used "gene-centered" high-throughput yeast one-hybrid (Y1H) assays to identify 283 interactions between 72 *C. elegans* digestive tract gene promoters and 117 proteins. The resulting protein-DNA interaction (PDI) network is highly connected and enriched for TFs that are expressed in the digestive tract. We provide functional annotations for ~10% of all worm TFs, many of which were previously uncharacterized, and find ten novel putative TFs, illustrating the power of a gene-centered approach. We provide additional in vivo evidence for multiple PDIs and illustrate how the PDI network provides insights into metazoan differential gene expression at a systems level.

INTRODUCTION

Differential gene expression is governed in part by the action of regulatory transcription factors (TFs) that bind *cis*-regulatory DNA elements that are often located in the promoters of target genes (Lee and Young, 2000). Of all metazoan genes, 5%–10% encode predicted TFs (Levine and Tjian, 2003). Each TF likely regulates multiple genes,

often in combination with other TFs, and may either activate or repress transcription. This combinatorial effect results in exquisitely fine-tuned spatial and temporal gene expression patterns and levels.

Physical interactions between TFs and their target genes can be visualized as protein-DNA interaction (PDI) networks that provide insights into differential gene expression at a systems level (Blais and Dynlacht, 2005). Superimposing the transcriptional consequence (i.e., activation or repression) of each PDI onto such networks allows the modeling of transcription regulatory networks that reveal the transcriptional logic underlying the system of study (Harbison et al., 2004; Levine and Davidson, 2005; Luscombe et al., 2004). A comprehensive understanding of the transcriptional mechanisms at a systems level requires the systematic identification of interactions between TFs and their target genes. Such PDIs can be identified using either TFs or their target genes as a starting point. To date, most studies have used "TF-centered" methods to find DNA sequences bound by TFs of interest (Blais and Dynlacht, 2005). These methods have mostly been applied to relatively simple systems such as yeast (Harbison et al., 2004; Horak et al., 2002) and mammalian cell lines (Cawley et al., 2004; Weinmann et al., 2002). It is technically challenging to systematically apply chromatin immunoprecipitation (ChIP)-based methods to metazoan systems, because TFs that are expressed at low levels, in a few cells, or during a narrow developmental interval are not suitable for such experiments. Moreover, antibodies are only available for few metazoan TFs. Thus, there is a need for complementary methods that are condition independent, amenable to high-throughput settings, and applicable to complex metazoan systems.

The yeast one-hybrid (Y1H) system is a "gene-centered" method that allows the identification of proteins that can bind to DNA sequences of interest (Li and Herskowitz, 1993; Wang and Reed, 1993). We recently developed

a Gateway-compatible version of the Y1H system, which allows the large-scale detection of PDIs (Deplancke et al., 2004). Here, we used this Y1H system to generate a first systematic PDI network of genes expressed and/or involved in the development and function of the *Caenorhabditis elegans* digestive tract.

Using 72 gene promoters as "DNA baits," we identified 283 PDIs that involve 117 proteins or "interactors." Most *C. elegans* TFs were heretofore uncharacterized. We provide a first set of putative target genes for ~10% of all predicted worm TFs and find ten novel potential TFs. We combine all PDIs into a network model. This network has similar properties as analogous networks in unicellular systems, indicating that the basic principles of transcription regulation at a systems level are evolutionarily conserved, and supporting our overall approach. The PDI network is enriched for TFs that are themselves expressed in the digestive tract, which further validates our approach. We provide estimates for the coverage of the Y1H system and additional experimental support for multiple PDIs. We illustrate how PDI network motifs can be converted into transcription regulatory network motifs that describe spatiotemporal aspects and transcriptional consequences of PDIs. Finally, we propose a model in which worm genes are under three layers of transcriptional control by a combination of different types of TFs: global, master, and specific regulators.

RESULTS

Y1H Assays

We selected a set of 167 *C. elegans* genes on the basis of one of the following criteria: (1) their expression is enriched in the developing pharynx (Gaudet and Mango, 2002); (2) their products or mammalian homologs are involved in endoderm development (Maduro and Rothman, 2002); or (3) they encode TFs that are expressed in the digestive tract (Table S1 in the Supplemental Data available with this article online). We reasoned that the inclusion of TF genes in this first gene set would allow us to start mapping a core PDI network (Davidson and Levin, 2005). We obtained 41 promoters from the promoterome (Dupuy et al., 2004) and cloned 126 ab initio. After cloning promoters into Y1H bait vectors, promoter::reporter constructs were sequentially integrated into the yeast genome. Thus promoters are packaged into chromatin which reduces the number of false positives (Deplancke et al., 2004). We obtained 116 promoter bait strains (Figure 1B). Six of these exhibited high levels of self-activation (Deplancke et al., 2004) and were omitted. Thus, in total, 110 promoters were used in Y1H assays (Table S1).

Each promoter bait strain was screened against a cDNA library and a TF mini-library (Figure 1A) (Deplancke et al., 2004). In total, we sequenced more than 3,200 Y1H positives to obtain interaction sequence tags (ISTs) (Walhout et al., 2000). To minimize the retrieval of false positives, we applied stringent filtering criteria (Figure 1C). In total, ~1,100 ISTs (~34%), corresponding to 209 PDIs, passed the filtering criteria. To test the specificity of the Y1H system, we tested all interactors available in the *C. elegans* ORFeome (Reboul et al., 2003) for binding to each of the 110 promoters by directed Y1H experiments. We identified 74 additional PDIs, out of more than 10,000 combinations tested, indicating that most PDIs retrieved are specific. In total, we identified 283 PDIs, involving 72 gene promoters and 117 interactors (Table S2).

The Y1H System Does Not Have an Inherent TF Bias

We classified interactors into TF families according to their annotated DNA binding domains in wTF2.0 (Reece-Hoyes et al., 2005). Importantly, we found members of most families (Table S2), indicating that the Y1H system does not have an inherent bias for or against certain types of TFs. For the majority of predicted worm TFs it has not been demonstrated that they can bind to DNA, and few TF targets have been reported. In addition, the biological function of most TFs remains elusive. Here, we provide DNA binding evidence and putative target genes for ~10% of the predicted worm TFs (Reece-Hoyes et al., 2005).

Y1H Assays: Coverage

To assess the coverage of our Y1H system, we investigated how many known TF-target gene interactions involving the 110 promoters were detected. Twelve PDIs between ten promoters and 11 TFs had been reported previously, underscoring the paucity of PDI data for *C. elegans* (Table S1). We retrieved four of these interactions (33%, Table S2). Although the numbers are low, this suggests that the Y1H coverage is similar to that of high-throughput yeast two-hybrid (Y2H) assays (Li et al., 2004) and in vitro PDI detection methods (Mukherjee et al., 2004). Two missed interactions involve PHA-4, a key regulator of pharynx development (Mango et al., 1994). However, we did find novel putative target genes for other master regulators of digestive tract development (e.g., ELT-2, Table S2).

The *C. elegans* PDI Network Has Similar Properties as PDI Networks from Unicellular Systems

We modeled all PDIs into a network using Cytoscape (Shannon et al., 2003) (Figure 2A). This network is highly connected as most nodes are connected and form one large network component. To further investigate the connectivity of the network, we plotted the outgoing connectivity, i.e., the number of promoter targets per interactor; and the incoming connectivity, i.e., the number of interactors per promoter (Luscombe et al., 2004) (Figures 2B and 2C). Interestingly, these connectivities follow distinct distributions. Whereas the outgoing connectivity follows a decaying power law distribution, the incoming connectivity follows a distribution closer to the exponential decrease of random networks. This observation is similar to what has been found in bacterial and yeast PDI networks (Guelzim et al., 2002). The average number of interactors per promoter in the PDI network is four, and varies between one and 14 (Figure 2C). In contrast, most interactors
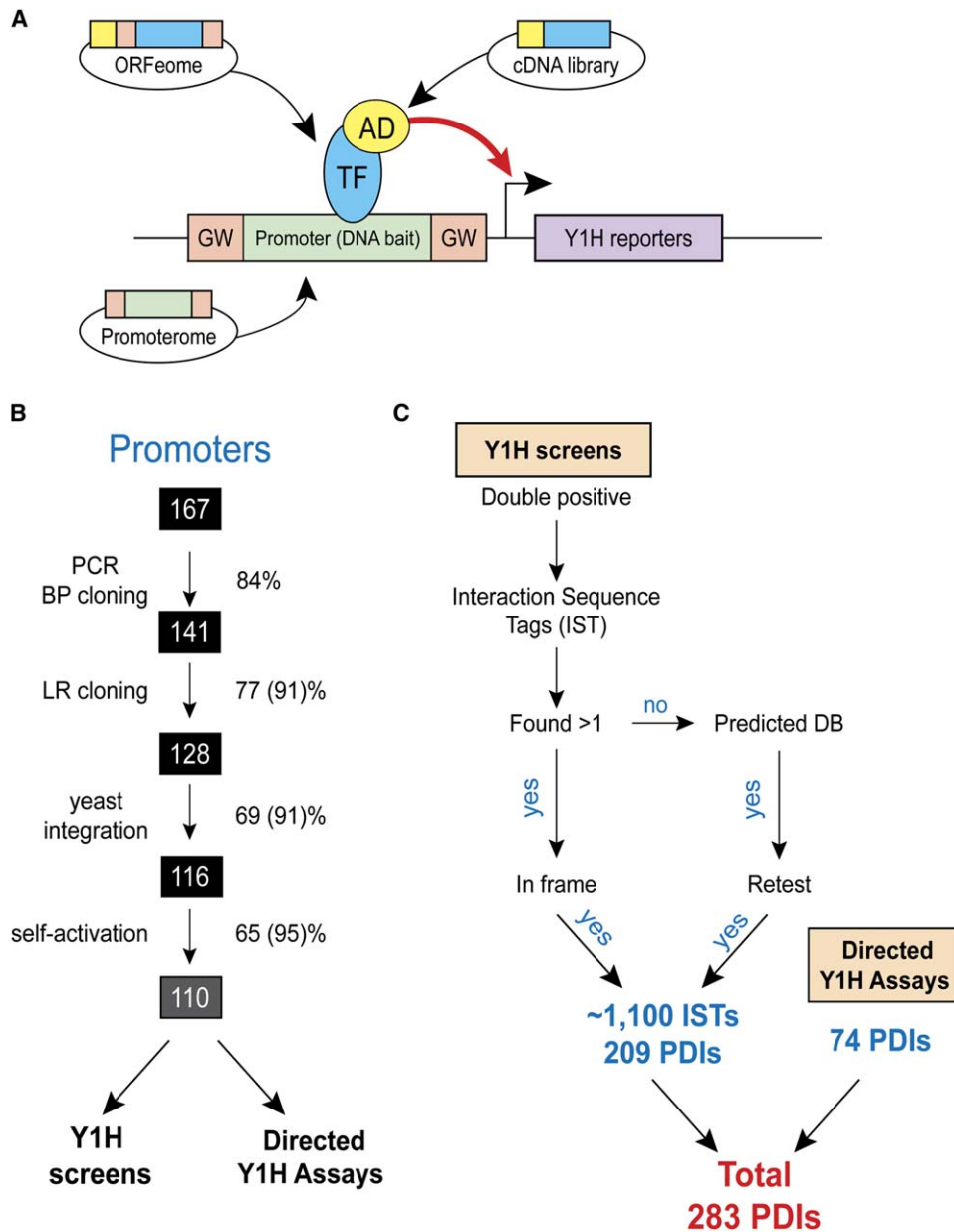
Figure 1. Y1H Assays and Pipeline

(A) Schematic overview of the Gateway-compatible Y1H system. GW = gateway recombination sites; AD = Gal4p activation domain. Promoters can be obtained from "promoterome" resources (Dupuy et al., 2004) and rapidly subcloned into Gateway Y1H "bait" vectors. TF-encoding open reading frames (ORFs) can be obtained from ORFeome resources (Reboul et al., 2003), cloned into a Y1H "prey" vector and pooled to create an AD-TF minilibrary (Deplancke et al., 2004). Two Y1H reporter genes, *HIS3* and *LacZ*, are used to prevent the inclusion of high numbers of false positives.

(B) Efficiencies of Gateway cloning steps and integration of promoter baits into the yeast genome. "PCR and BP cloning" refers to promoters that were obtained by PCR using *C. elegans* genomic DNA as a template and that were successfully cloned to create promoter Entry clones. LR cloning refers to the cloning of promoters from Entry clones into *HIS3* and *LacZ* Y1H reporter Destination vectors. Yeast integration refers to the integration of promoter::reporter constructs into the yeast genome. Self-activation refers to the activation of reporter gene expression in the absence of an exogenous *C. elegans* interactor. Percentages refer to the cumulative success rate and the percentages per step are indicated in parentheses.

(C) Y1H interactions were only considered if they conferred a positive readout for both Y1H reporter genes, were found several times per promoter, or retested in fresh promoter bait strains. In addition, preys that do not have an annotated DNA binding domain were removed, except if they were found multiple times. Finally, ISTs that were not in the correct frame were removed.

interact only with one or two promoters (84%), but some TFs bind a relatively large number of promoters (up to 27, Figure 2B). This suggests that well-connected TFs provide a high degree of connectivity to the network. Indeed, 71% of the promoters in the network are bound by at least one of the top 10% most connected interactors,
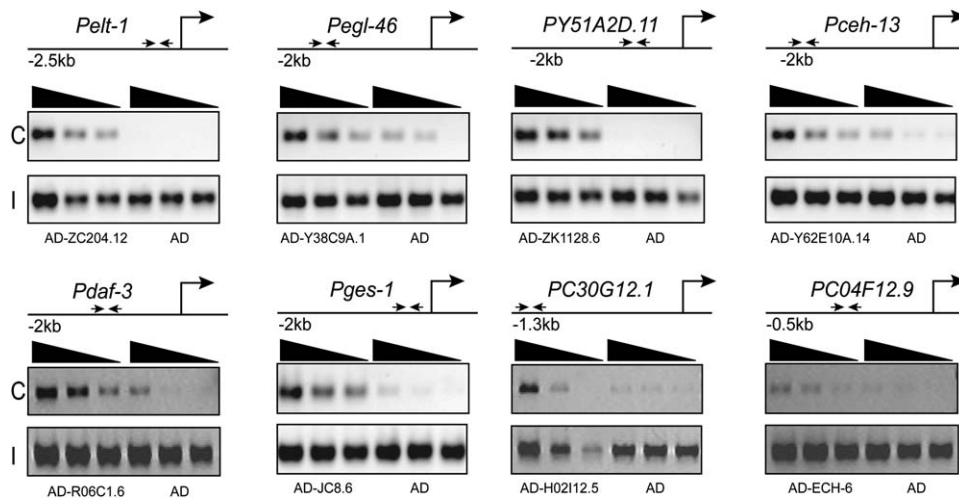
A

D

B outgoing

C incoming
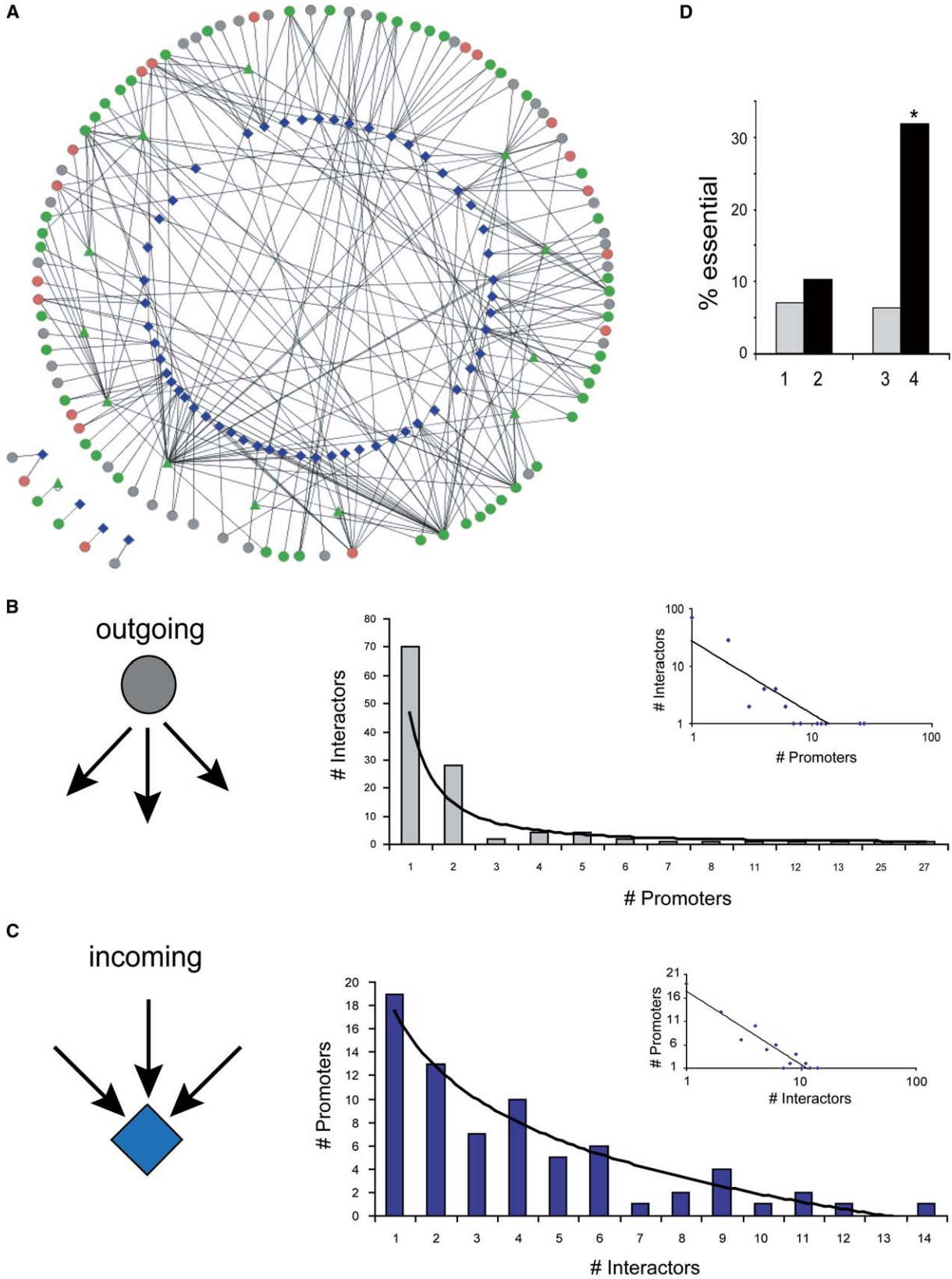
Figure 3. Confirmation of DNA Binding of Novel Putative TFs by ChIP in Yeast
Each panel shows the promoter with the amplified region indicated by arrows at the top and the ChIP results, using an anti-Gal4p activation domain (AD) antibody on the bottom. C, ChIP product; I, input PCR product. AD, Gal4 activation domain. AD alone was used as a negative ChIP control. For each novel putative TF, one target promoter was examined. Black triangles indicate 3-fold titration steps of input and sample template.

suggesting that these interactors may represent "global" regulators of transcription (see Discussion). We expect that the number of promoters bound by these well-connected interactors will increase as additional Y1H interactions become available.

In yeast, well-connected TFs are more likely to be essential than less well-connected TFs (Yu et al., 2004). We tested whether a similar relationship exists in the *C. elegans* PDI network. We determined the percentage of essential proteins for interactors that bind three or more promoters (16%) and for those that bind only one or two promoters (84%). We found that 32% (6/19) of interactors that bind three or more promoters and 6% (6/98) of interactors that bind one or two promoters are essential, respectively (p < 0.001, Figure 2D). A similar enrichment for essentiality was observed when cut-offs of four and more, five and more targets, etc. were chosen (data not shown). Thus, there is a positive correlation between connectivity and essentiality for well-connected TFs in both unicellular and metazoan systems. Next, we analyzed the PDI network for the presence of network motifs that are significantly overrepresented compared to 100 randomized networks with the same single-node characteristics and found several motifs that were previously shown also to be enriched in bacterial and yeast PDI networks (Table S3) (Milo et al., 2002; Shen-Orr et al., 2002).

Taken together, the Y1H-based *C. elegans* PDI network has similar properties and motifs as PDI networks of relatively simple unicellular systems, providing additional confidence to the quality of our data. It should be noted, however, that our network is a relatively small sample of the entire worm PDI network and that 51% of the promoters used correspond to TF-encoding genes, whereas only ~5% of all *C. elegans* genes encode predicted TFs (Reece-Hoyes et al., 2005). Thus, in the future, it will be important to investigate the properties and motifs of more complete metazoan PDI networks.

The Y1H System Can Be Used to Discover Novel DNA Binding Proteins
Ten interactors (8.5%) do not possess a known DNA binding domain yet pass the filtering criteria. Thus, we hypothesized that these may be novel TFs. To test if these proteins directly bind their target promoters in yeast, we performed ChIP assays using an anti-Gal4AD (activation domain) antibody. We confirmed DNA binding for eight of the ten novel putative DNA binding proteins (80%, Figure 3). This demonstrates that the Y1H system provides a powerful tool to discover novel putative TFs, which is a unique advantage of gene-centered approaches.
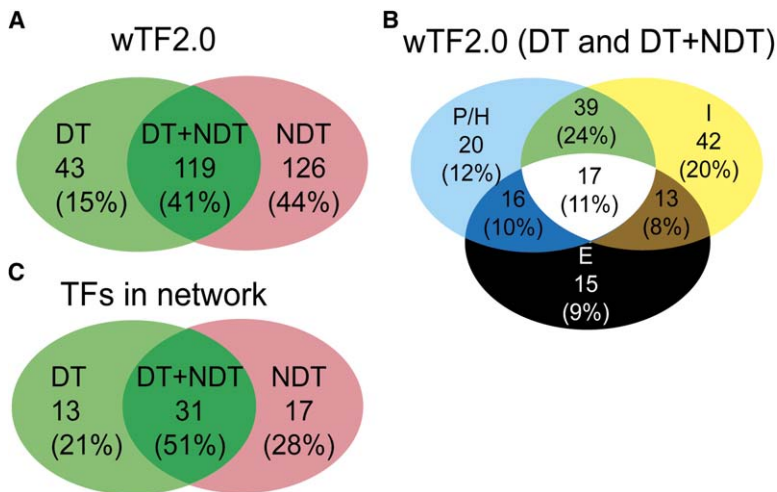
Figure 2. A PDI Network of *C. elegans* Digestive Tract Genes
(A) Y1H interactions were modeled into a PDI network using Cytoscape 2.2 (Shannon et al., 2003). Blue diamonds, promoters; circles, interactors; triangles, interactors whose promoters were also used as DNA baits. Green, interactors with a digestive tract annotation; red, interactors that are not in the digestive tract; gray, all other interactors. PDIs are depicted as black lines.
(B) The departing connectivity follows a decaying power law distribution (R = 0.86). Inset: log-log scale graph.
(C) The arriving connectivity follows a logarithmic distribution (R = 0.95). Inset: semi-log scale graph.
(D) Interactors that bind to three or more promoters have a higher likelihood of being essential than those that bind to one or two promoters. 1, all *C. elegans* genes; 2, all interactors in the PDI network; 3, interactors that bind one or two promoters; 4, interactors that bind three or more promoters. Asterisk, significant enrichment (p < 0.001).

The PDI Network Is Enriched for Digestive Tract TFs

Systematic data integration can be used to assess the quality of functional genomic data and to provide degrees of confidence to individual data points (Ge et al., 2003). We hypothesized that, by starting with a set of digestive tract gene promoters, we would retrieve many TFs that are themselves expressed in this system. To test this, we integrated the PDI network with available expression pattern data. We found expression pattern information for 288 of the 934 predicted TFs (Reece-Hoyes et al., 2005). Of these, 162 (~56%) are expressed in the digestive tract, but only 43 are exclusively found in this system (Figure 4A, Table S4), suggesting that many TFs play a relatively pleiotropic role in gene expression. Interestingly, of the 148 TFs expressed in the digestive tract in larvae and adults, 38% (56/148) are expressed in both the pharynx *and* the intestine, even though these parts are derived from distinct germ layers (Figure 4B). We found expression pattern information for 61 TFs in the network (excluding potential novel TFs), and of these, 44 (72%, p < 0.01) are expressed in the digestive tract (Figure 4C, Table S2). Next, we determined the expression pattern for 14 uncharacterized interactors and found that 12 of these (86%) are also expressed in the digestive tract (Figures S1 and S2, see also Figure 6B). Together, these data demonstrate that the PDI network is significantly enriched for TFs expressed in the digestive tract, which further supports our overall approach.

Four of the 14 interactors for which we determined the expression pattern are among the most highly connected nodes in the network as they bind to many target promoters (i.e., ZTF-1, ZC204.12, JC8.6, and ZTF-2). Interestingly, worms that contain translational *PZC204.12* constructs (i.e., in which GFP is expressed as a fusion protein with ZC204.12) are slow growing and thin, and worms containing translational fusions of *ztf-1* or JC8.6 could not be stably maintained (data not shown). Further, the expression of ZTF-1::GFP fusions is restricted to a few head neurons in adult animals, while GFP is more broadly expressed and throughout development in animals carrying a transcriptional *Pztf-1* fusion (Figure S1B). This is consistent with endogenous *ztf-1* expression as its mRNA has been detected in the developing embryo by microarray analysis (Baugh et al., 2003). Together, these data suggest that overexpression of putative global regulators may be toxic because of changes in the expression of a large number of genes (see Discussion).

Finally, we superimposed all expression pattern information onto the PDI network (Figure S3A). The resulting integrated network can be used to derive subgraphs that provide hypotheses about where particular PDIs may function in the animal (examples are shown in Figures S3B and S3C). Several interactors in the network do not appear to be expressed in the digestive tract. Such interactors may correspond to false positives or, alternatively, may function to prevent expression of digestive tract genes in other tissues.

Transcriptional Consequences of PDIs

In Y1H assays, TFs are fused to a heterologous transcription activation domain (Figure 1A). Thus, Y1H assays do not provide insight into the transcriptional function of TFs. To initiate the analysis of the transcriptional consequences of individual PDIs, we first used quantitative RT-PCR. We obtained worm deletion strains for eight TFs and compared expression of their 16 Y1H targets between TF deletion strains and wild-type animals. We found gene expression changes for three PDIs (19%) involving the interactors DAF-3, DAF-16, and ELT-3 (Figure 5A and data not shown). This is likely an underestimation of the proportion of genuine in vivo interactions as we used mixed populations of animals and because PDIs may occur in only a few cells or at narrow developmental intervals. We also tested whether the expression of the other genes (i.e., those not found as targets by Y1H) was affected in the deletion strains and found no changes. This indicates that PDIs found by Y1H assays are specific. Interestingly, all three interactors repress transcription of their Y1H targets. This is consistent with previous
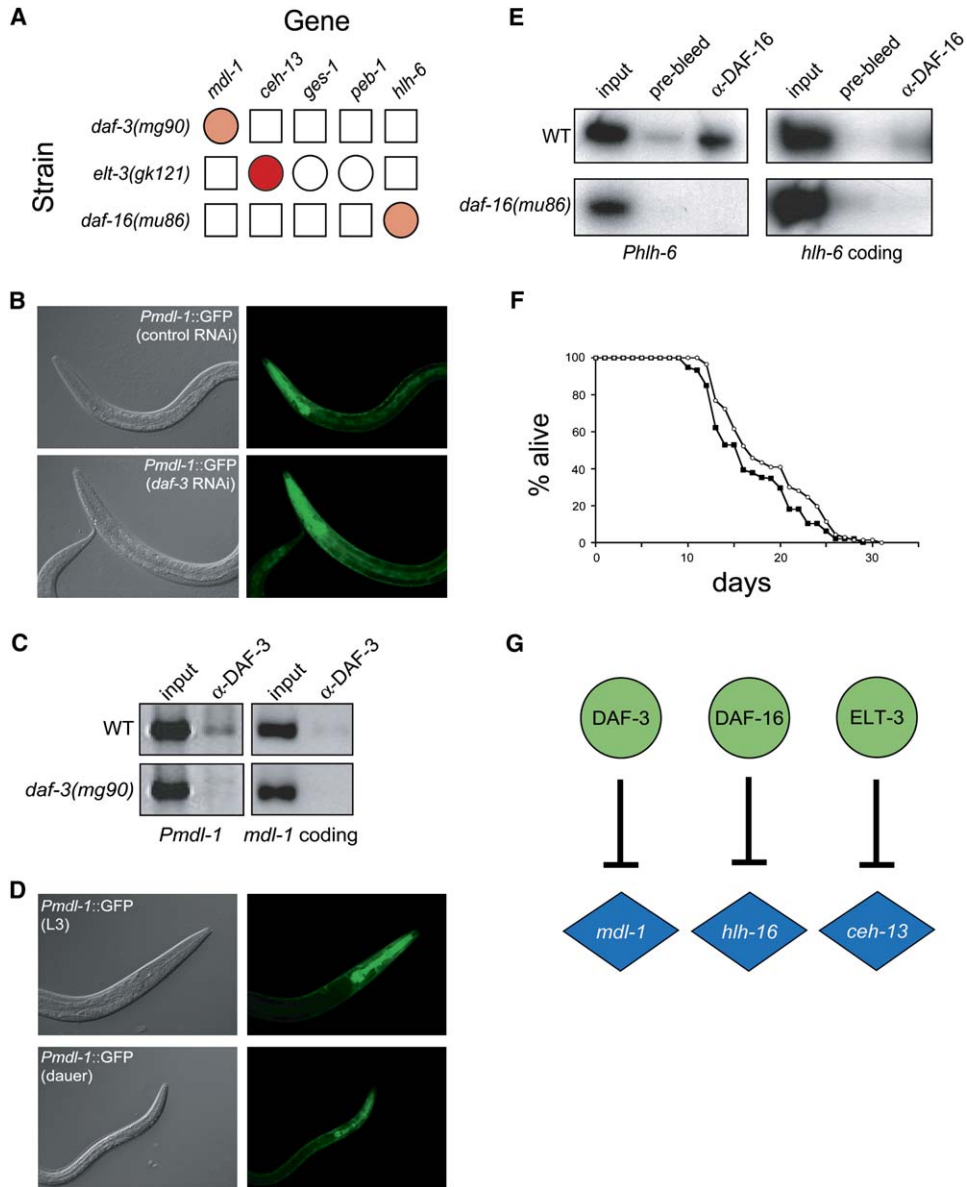
Figure 5. Validation of Individual PDIs

(A) Quantitative RT-PCR shows that DAF-3, ELT-3, and DAF-16 repress the expression of their Y1H targets *mdl-1*, *ceh-13*, and *hlh-6,* respectively. Changes are expressed as fold increases compared to wild-type worms. Circles, Y1H-detected interactions; squares, negative controls; light red indicates 2- to 4-fold increase; and red indicates >4-fold increase. White indicates no change in expression.

(B) *mdl-1* promoter activity in *daf-3(RNAi)* and control(RNAi) animals. Left panels, DIC images; right panels, GFP fluorescence.

(C) DAF-3 was chromatin immunoprecipitated with anti-DAF-3 antibody from either wild-type or *daf-3(mg90)* mutant animals. Input, PCR control; *mdl-1* coding, negative control.

(D) *mdl-1* pharyngeal promoter activity decreases when worms enter the dauer stage. This effect was observed in 100% of the animals. Left panels, DIC images; right panels, GFP fluorescence.

(E) DAF-16 was chromatin immunoprecipitated with either anti-DAF-16 antibody or preimmune serum (prebleed) from either wild-type worms or *daf-16(mu86)* animals. Input, PCR control; *hlh-6* coding, negative control.

(F) Life spans of *hlh-6(RNAi)* (n = 203) or vector RNAi (n = 193) animals are plotted. Average life spans were 16.9 ± 0.35 days for control and 18.6 ± 0.36 days for *hlh-6(RNAi)* worms (p < 0.001). Life span assays were performed five times. Averaged data for all experiments are shown. Black squares, wild-type; open circles, *hlh-6* RNAi.

(G) PDIs can be converted into transcription regulatory interactions.

observations for DAF-16 and DAF-3 in *C. elegans* (Oh et al., 2006; Thatcher et al., 1999) or ELT-3 homologs in other systems (Schwenger et al., 2001).

Quantitative RT-PCR does not provide insights into where and when changes in target gene expression occur. To address this issue, we created transgenic worms that express GFP under the control of the promoter of *mdl-1* (*Pmdl-1*::GFP), a Y1H target of DAF-3. These animals express GFP in both the intestine and the pharynx, as well as in the hypodermis and ventral nerve cord (Figure 5B and data not shown). Consistent with the quantitative RT-PCR results, we found that knocking down *daf-3* by RNAi results in an increase in *mdl-1* promoter activity. Interestingly, this effect specifically occurs in the pharynx (Figure 5B). The Y1H interaction between DAF-3 and *Pmdl-1* suggests that *mdl-1* is a direct DAF-3 target. To test this, we used an anti-DAF-3 antibody in ChIP assays and found that endogenous DAF-3 is indeed localized to the endogenous *mdl-1* promoter (Figure 5C). DAF-3 promotes *C. elegans* dauer formation (Patterson et al., 1997). Interestingly, in a microarray study comparing dauer to nondauer worms, Wang and Kim (2003) found that *daf-3* mRNA is up-regulated ∼40-fold when wild-type worms enter the dauer stage, whereas *mdl-1* mRNA is downregulated up to ∼100-fold. We confirmed that pharyngeal *mdl-1* promoter activity is indeed dramatically decreased in dauers (Figure 5D and Table S5). Taken together, our data strongly suggest that *mdl-1* is a direct in vivo target of DAF-3 and that DAF-3 represses *mdl-1* gene expression in the pharynx when worms enter the dauer stage.

DAF-16 is central to the regulation of dauer formation, aging, and fat storage (Patterson, 2003). We found that endogenous DAF-16 directly binds to its Y1H target, *hlh-6*, in vivo (Figure 5E), and therefore tested whether *hlh-6* is involved in any of these biological processes as well. Whereas *hlh-6(RNAi)* does not affect dauer formation or fat storage (data not shown), it does result in a modest (10%) but reproducible and significant (p < 0.001) extension of lifespan (Figure 5F). *daf-16* loss-of-function mutants have a reduced lifespan (Lin et al., 1997). Thus, the observation that *hlh-6(RNAi)* results in lifespan extension is in agreement with the finding that DAF-16 represses *hlh-6* expression.

Taken together, these results demonstrate how we can integrate expression pattern information together with the transcriptional consequences of individual PDIs as a first step toward the characterization of transcription regulatory networks (Figure 5G).

Converting PDI Network Motifs into Transcription Regulatory Network Motifs

The *C. elegans* digestive tract is composed of different tissues and cell types and it is likely that only parts of the PDI network are relevant under particular (developmental) conditions or in particular cells. To initiate the conversion of PDI motifs into transcription regulatory network motifs that, in addition to revealing the transcriptional consequences of PDIs, provide spatial and temporal information

of PDIs, we focused on a single input motif in which ZTF-2 binds five pharyngeal gene promoters (Figure 6A). Because we did not detect ZTF-2 binding to intestinal gene promoters, we hypothesized that ZTF-2 may be a regulator of pharyngeal gene expression. To test this, we first examined the *ztf-2* expression pattern and found that it is expressed in the pharynx (Figures 6B and S2). Next, we tested whether removal of ZTF-2 results in changes in activity of its Y1H target, *Pfat-5*, which weakly drives expression of GFP throughout the digestive tract, with strongest expression in the pharynx (Figure 6C and data not shown). Knocking down *ztf-2* by RNAi in *Pfat-5*::GFP containing animals resulted in an increase of GFP expression, suggesting that ZTF-2 represses pharyngeal gene expression (Figure 6C, Table S6). Knocking down two other *Pfat-5* interactors (DIE-1 and EGL-44) by RNAi resulted in a small decrease in embryonic GFP expression, indicating that these TFs may be positive regulators of *fat-5* gene expression (Table S6 and data not shown). Next, we analyzed the promoters of the ZTF-2 targets for overrepresented DNA elements and found an element that is very similar to a previously described *cis*-regulatory pharyngeal element ("P2," Figure 6D) (Gaudet et al., 2004). ZTF-2 can bind P2 in the Y1H system, suggesting that it does constitute a ZTF-2 binding site (Figure 6E). Finally, we investigated if ZTF-2 can bind P2 in vivo and if ZTF-2 activates or represses transcription through this element. We used a transgenic worm strain containing three copies of P2, linked to a minimal *pes-10* promoter and driving GFP expression (Gaudet et al., 2004). We compared GFP expression in wild-type and *ztf-2(RNAi)* animals. As reported previously, P2 drives weak expression in the developing pharynx (Figure 6F) (Gaudet et al., 2004). Knocking down *ztf-2* did not change the pattern of GFP expression, but did result in increased GFP levels in the pharynx (Figure 6F, Table S7), indicating that P2 is indeed a functional ZTF-2 binding site, and confirming that ZTF-2 represses pharyngeal gene expression. Interestingly, we observed a low penetrance Pun (*pharynx unattached*) phenotype in *ztf-2(RNAi)* animals (Figure 6G, Table S7). This finding is consistent with the observation that ZTF-2 is expressed in the pharyngeal epithelium/arcade, which connects the pharynx to the mouth (Figure 6B), and suggests a role for ZTF-2 in pharynx morphogenesis. Taken together, these results demonstrate how a combination of computational and experimental methods can be used to convert a PDI network motif into a transcription regulatory network motif (Figure 6H).

DISCUSSION

Gene-Centered Y1H Assays Provide a Powerful Complementary Method for the Mapping of Transcription Regulatory Networks in Complex Metazoan Systems

TF-centered PDI studies in intricate metazoan systems are hampered by the lack of suitable antibodies and the inaccessibility of many TFs. Indeed, 934 worm genes
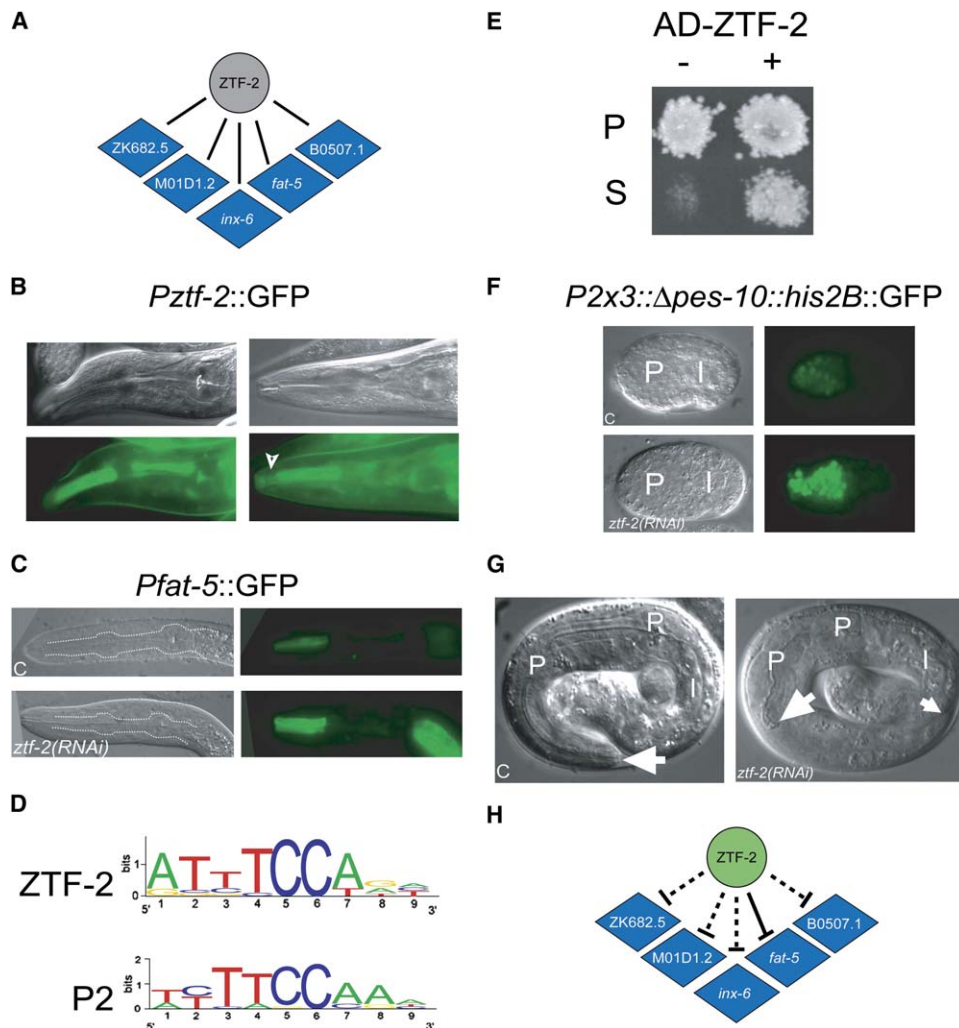
Figure 6. Converting PDI Network Motifs into Transcription Regulatory Network Motifs

(A) The ZTF-2 single input network motif. Blue diamonds, target promoters; black lines, Y1H PDIs.

(B) The *ztf-2* promoter is active in the pharynx. DIC images and GFP fluorescence of two animals are shown. White indicates GFP expression in the arcade.

(C) ZTF-2 represses *fat-5* gene expression in L1 larvae and embryos. Transgenic worms containing *Pfat-5*::GFP were injected with *ztf-2* dsRNA or control dsRNA (C), and the corresponding GFP expression patterns and levels were compared. White dotted lines outline the pharynx.

(D) Using the motif finder algorithm Improbizer (Ao et al., 2004), we identified an overrepresented DNA element in the ZTF-2 Y1H target promoters (upper WebLogo). This element exhibits strong similarity to a known pharyngeal element (P2, lower WebLogo; Gaudet et al., 2004).

(E) A yeast bait strain containing six copies of P2 fused to the *HIS3* Y1H reporter gene was transformed with AD-ZTF-2 (+) or empty vector plasmid (−). P, permissive media; S, selective media lacking histidine and containing 3AT.

(F) Transgenic worms containing a 3XP2::Δ*pes-10*::GFP::*his2B* construct (Gaudet et al., 2004) were injected with *ztf-2* dsRNA, or control dsRNA (C), and the corresponding GFP expression patterns and levels were compared. *his2B* encodes a nuclear histone and is used for nuclear visualization of GFP. Embryos in which the pharynx (P) and intestinal (I) primordium is being formed are shown. Left, DIC images; right, GFP fluorescence.

(G) Loss of ZTF-2 by RNAi results in a Pun (*p*harynx *un*attached) phenotype. DIC images of 3-fold wild-type (WT) and *ztf-2(RNAi)* embryos. The arrows indicate the mouth and pharynx.

(H) The PDI network motif can be converted into a transcription regulatory network motif, and the ZTF-2 node can be colored green. Solid line, confirmed repression of *fat-5* expression by ZTF-2; dashed lines, inferred repression by ZTF-2 of its other Y1H targets.

encode TFs (Reece-Hoyes et al., 2005), but very little information regarding their DNA binding sites and target genes had been reported. Gene-centered approaches have some unique advantages for PDI mapping. For instance, we provide functional annotations for ∼10% of all predicted *C. elegans* TFs. In addition, we find ten novel potential TFs and show that eight of these are likely genuine DNA binding proteins. Recently, a yeast enzyme (Arg5,6)

has been shown to specifically bind to DNA (Hall et al., 2004), supporting the notion that not all DNA binding domains have been uncovered.

As with any large-scale method, it is important to estimate both the sensitivity and specificity of the Y1H assay. We detect ∼33% of previously reported PDIs, which is similar to the coverage of other PPI and PDI data sets (Li et al., 2004; Mukherjee et al., 2004). There are several reasons why we may miss interactions: (1) Currently, the Y1H system is not configured to identify heterodimers, which explains why we did not find the interaction between Pceh-24 and HLH-8/HLH-2. (2) The Y1H screens are not saturated. This is perhaps best illustrated by the identification of 74 additional PDIs by directed Y1H experiments. (3) As we use gene promoters as DNA baits, we miss interactions that occur in other regions (e.g., introns). (4) TFs that need to be posttranslationally modified in order to bind DNA will likely not be detected.

There are two classes of false positives in Y1H assays: those that are the result of technical limitations, and those that are reproducible in Y1H assays but are not relevant in vivo. To avoid the inclusion of "technical false positives," we applied stringent filtering criteria that have been shown to greatly reduce the number of false positives in Y2H assays to the Y1H data. Moreover, as we mostly expect to find TFs, we mostly include proteins that possess a predicted DNA binding domain. We assess the quality of the filtered Y1H data in several ways. For instance, using digestive tract gene promoters, we find more TFs that are expressed in this system than expected by chance. In addition, we use a variety of methods including ChIP, quantitative RT-PCR, and RNAi to assess the in vivo relevance of several PDIs.

Multiple members of one TF family sometimes bind to a single promoter (e.g., GATA-type zinc finger TFs, Table S2) and may have similar DNA binding specificities. Indeed, it has been reported that several GATA TFs are able to bind similar sequences (Ko and Engel, 1993). Alternatively, multiple members of a TF family may truly regulate the expression of a single gene in vivo, for example by binding to the same cis-regulatory element under different circumstances, or by binding to nonoverlapping, co-occurring binding sites, as has been described for nuclear hormone receptors (Chawla et al., 2001). Recently, Pauli and co-workers have shown that multiple GATA TFs regulate the expression of overlapping C. elegans genes in vivo (Pauli et al., 2006). However, based on Y1H data alone, we cannot discern whether multiple TFs bind to a target promoter simultaneously or if these TFs bind individually in different cells and/or at different developmental times.

Taken together, gene-centered Y1H assays provide a powerful alternative for the systematic identification of PDIs to TF-centered methods such as ChIP in C. elegans. However, as both TF-centered and gene-centered methods have different limitations and advantages, it will be important to apply both types of methods to attain optimal coverage and quality in PDI datasets.

Converting Protein-DNA Interaction Networks into Transcription Regulatory Networks

PDI networks are static models in which all interactions that may occur during the development, function and, perhaps pathology, of a system are combined. We illlustrate how several approaches can be used to convert static PDI network motifs into transcription regulatory network motifs that describe transcriptional consequences of PDIs and spatiotemporal aspects of gene regulation. First, we show that quantitative RT-PCR can be used to determine whether target gene expression is up –or downregulated when a TF that binds to the target's promoter in Y1H assays is removed. Second, both transcriptional consequences of PDIs and spatiotemporal information of PDIs can be inferred in transgenic animals in which GFP is expressed under the control of a target gene promoter. This is done by comparing GFP levels and patterns between wild-type animals and animals in which TF levels are knocked down by RNAi. Interestingly, we find that several TFs repress expression of their targets, even though these targets are themselves expressed in the digestive tract. This suggests that these TFs function to fine-tune gene expression levels rather than to determine gene expression patterns.

A Model for C. elegans Differential Gene Expression at a Systems Level

We find that most TFs bind only one or two promoters, whereas a few TFs bind many (Figure 2B). On average, each promoter is bound by four TFs, and more than 70% of the promoters are bound by at least one of the top 10% most highly connected TFs. In addition, 82% of the promoters are bound by at least one of the other less-well connected interactors, and more than half of our target promoters bind both. Together, these observations lead to a model in which C. elegans genes are subjected to three or more layers of transcriptional control (Figure 7). The first layer consists of global regulators that control the expression of many genes in many different systems (e.g., DIE-1, ZTF-1, JC8.6, and ZC204.12, which bind both pharyngeal and intestinal gene promoters). Our preliminary data indicate that these putative global regulators also appear as highly connected interactors in other, unrelated C. elegans PDI networks (data not shown). The second layer involves "master regulators" that control the expression of multiple genes that are specifically involved in a particular system (e.g., PHA-4 in the pharynx and ELT-2 in the intestine) (Labouesse and Mango, 1999; Fukushige et al., 1998). Finally, the third layer constitutes "specifiers" that fine-tune the expression of a relatively small number of genes. In agreement with this model, well-connected interactors have a relatively high likelihood to be essential, whereas less well-connected interactors do not (Figure 2D). Based on this model, we predict that metazoan transcription regulatory networks will possess a hierarchy that reflects these layers. Interestingly, bacterial transcription regulatory networks also exhibit a layered hierarchy as many genes are
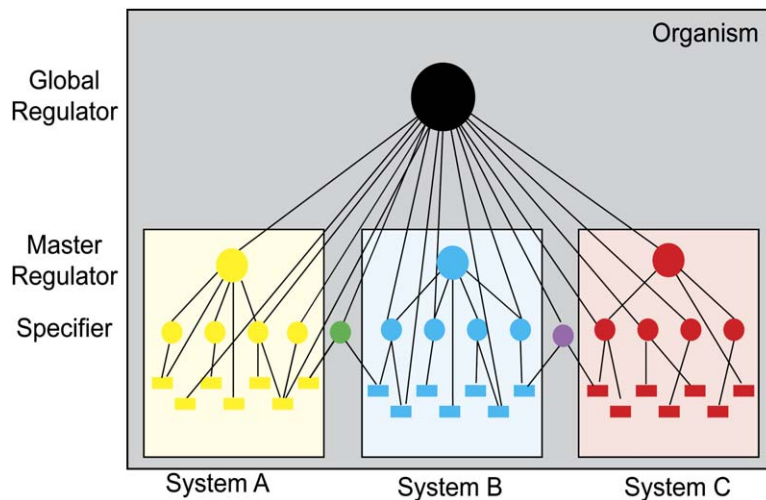
Figure 7. A Model for *C. elegans* Differential Gene Expression

Based on our PDI network data, we propose a hierarchical model in which most *C. elegans* genes are regulated by a combination of three or more types of TFs: Global regulators that bind many promoters from different systems, master regulators that bind many promoters within a system and that are indispensable for system development, and specifiers that fine-tune gene expression. Yellow, blue, and red indicate different systems. The green and purple nodes illustrate that specifier TFs may also be found in multiple systems.

regulated by a combination of a handful of global regulators and multiple specifiers (Martínez-Antonio and Collado-Vides, 2003). The *C. elegans* PDI network contains similar motifs as bacterial networks (Shen-Orr et al., 2002). Together, this suggests that the principles controlling gene expression at a systems level are evolutionarily conserved and extend beyond the single cell level. The availability of additional metazoan PDI data will reveal whether this model reflects the general principles underlying differential gene expression in *C. elegans* and other higher eukaryotes, including humans. Y1H assays provide a method to further test this model in other *C. elegans* systems, and this study may serve as a blueprint for similar studies in higher organisms as well.

EXPERIMENTAL PROCEDURES

Generation of Y1H Promoter Bait Strains

Promoters were cloned into Y1H reporter Destination vectors by Gateway cloning and integrated into the genome of YM4271 yeast as described elsewhere (Deplancke et al., 2004; B.D. and A.J.M.W., unpublished data). Bait strains were verified by PCR of yeast genomic DNA using vector-specific primers, after which PCR amplicons were sequenced. Self-activation of promoter bait strains was tested as described (Deplancke et al., 2004). Promoter bait strains that exhibited growth on Sc-His,-Ura media containing 3-aminotriazole (3AT) concentrations of 80 mM or higher were omitted (Table S1).

Y1H Screens

Promoter bait strains were transformed with the AD-wrmcDNA and AD-TF libraries as described (Deplancke et al., 2004), and plated onto Sc-His,-Ura,-Trp media containing appropriate concentrations of 3AT. A minimum of 1×10^6 and 3×10^5 colonies were screened per strain for the AD-wrmcDNA and AD-TF libraries, respectively. Potential positives were picked and tested for β-galactosidase expression as described (Deplancke et al., 2004). Yeast colony PCR was performed as described (Walhout and Vidal, 2001). Sequencing was performed by Agencourt Bioscience Corporation, and interactors were identified by BLAST. Interactor sequences are referred to as Interaction Sequence Tags (ISTs) (Walhout et al., 2000).

Directed Y1H Experiments

Two types of directed Y1H experiments were performed: (1) mating experiments using strains of opposite mating types, one containing the promoter bait, and the other containing the AD-TFs (B.D. and A.J.M.W., unpublished data), and (2) direct transformations of 100 ng AD-TF plasmid into promoter bait strains (DIE-1, JC8.6). Mating was performed as described (Walhout and Vidal, 2001).

Identification of "Literature" Interactions

PubMed inquiries were done using both the ORF and gene name of each of the 110 successfully obtained promoters. In addition, we surveyed WormBase (Chen et al., 2005) for reported PDIs.

Analysis of Network Properties

The most suitable distributions for the departing and arriving connectivities were found by determining the best-fitting exponential ($p(k) \sim e^{-\gamma k}$), logarithmic ($p(k) \sim -C*Ln(k) + \gamma$), and power-law ($p(k) \sim k^{-\gamma}$) distributions, where $p(k)$ represents the fraction of interactor or promoter nodes, and k the number of interactions. The best-fitting distribution was determined by minimizing the root mean squared difference (RSD) between the actual and fitted distributions as obtained by scatter plot analysis, and by comparing the regression value R for each distribution. Departing connectivity followed a decaying power law distribution ($\gamma = -1.27$) and the RSD and R for this distribution equal 12.8 and 0.86, compared to 13.1 and 0.74 for a logarithmic, and 19.9 and 0.6 for an exponential distribution. Arriving connectivity followed a logarithmic distribution ($\gamma = 17.4$) as the RSD and R for this distribution equals 1.7 and 0.95 respectively compared to 2.1 and 0.88 for the exponential, and 3.2 and 0.88 for the power-law distributions. Motif analysis was performed using Mfinder as described (Shen-Orr et al., 2002).

RNAi Phenotype Analysis
See Supplemental Data.

Yeast ChIP

Yeast ChIP was performed as described previously (Harbison et al., 2004) using 3 μl anti Gal4-AD antibody (GAL4-TA, Santa Cruz). Each experiment was performed in duplicate, and repeated twice. PCR promoter scanning in fragments of ~300 bp was performed. Fragments which yielded the greatest difference in PCR amplicon intensity between the novel interactor-containing yeast cells and the negative control (AD only) for both duplicates were titrated in three 2-fold steps as shown in Figure 3. The amplification protocol included one cycle of 2 min at 95°C, 33–36 cycles of three steps each: 30 s at 95°C, 60 s at 56°C, and 30 s at 68°C. Primer sequences are available in Table S8.

Identification of TFs with a Digestive Tract Annotation

Only interactors that possess a predicted DNA binding domain were considered. To find expression pattern information for all predicted TFs, we used the database query tool WormMart (version WS148, http://www.wormbase.org/biomart/martview). We found expression pattern information for 288 of the 934 predicted TFs (Reece-Hoyes et al., 2005). We manually curated the list of 288 TFs to annotate TFs that are expressed in the digestive tract. Only TFs expressed in structural parts of the digestive tract (i.e., the musculature, glands and epithelium) were included. In addition, we included TFs that are expressed in embryonic lineages that give rise to the pharynx, intestine, or hindgut, where specifically indicated. 162 of 288 (56%) TFs are expressed in the digestive tract (Table S4). We found expression pattern information for 61 TF interactors, of which 44 (72%) are expressed in the digestive tract. A binomial distribution (n = 61, p = 0.56, k $\geq$ 44) was used to calculate the enrichment significance (p < 0.01). We also retrieved mutant phenotypes and gene expression profiles of all interactors (Chen et al., 2005). Interactors involved in digestive tract development or enriched in the developing pharynx were given a digestive tract annotation in the PDI network, but were not included in the enrichment calculations. We found that 50 of 117 (43%) interactors have a digestive tract annotation, 17 (15%) do not, and the other interactors (42%) are not yet functionally annotated (Table S2).

Expression Pattern Analysis
See Supplemental Data.

Quantitative RT-PCR
See Supplemental Data.

Worm ChIP

DAF-16 ChIP analysis was performed as described (Oh et al., 2006). PCR amplification was carried out in the presence of ^{32}P-labeled dATP, after which the amplicons were resolved on a polyacrylamide gel. DAF-3 ChIP analysis was performed using an anti-DAF-3 antibody (N.M. and A.J.M.W., unpublished data) and amplicons were resolved on a 2% agarose gel. Primer sequences are available in Table S8.

Life Span Analysis

Life span assays were performed 5 times at 20ºC as described (Mukhopadhyay et al., 2005).

Generation of *Pfat-5*::GFP and *Pmdl-1*::GFP Transgenic Worms
See Supplemental Data.

Generation of the 6xP2 Element Y1H Construct

To generate a DNA bait construct containing six copies of the P2 element, two pairs of complementary oligonucleotides (Table S8), each containing three P2 copies, were annealed, ligated via a CACA bridge, and cloned into pUC19. M13 primers flanked by Gateway-compatible cloning sites were used to PCR amplify the 6xP2 fragment, after which the resulting PCR amplicon was cloned via a Gateway BP reaction into the Entry vector pDONR-P4-P1R as described (Dupuy et al., 2004). One sequence-verified P2 multimer Entry clone was then used for LR cloning into the Y1H destination vectors as described (Deplancke et al., 2004).

RNA Interference of Y1H Interactors
See Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, three figures, Supplemental References, and eight Excel spreadsheets and can be found with this article online at http://www.cell.com/cgi/content/full/125/6/1193/DC1/.

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