Current Biology *17*, 1635–1645, October 9, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.08.058

Article

The *C. elegans* TGF-β Dauer Pathway Regulates Longevity via Insulin Signaling

Wendy M. Shaw,[1,2] Shijing Luo,[1,2] Jessica Landis,[1,2] Jasmine Ashraf,[1,2] and Coleen T. Murphy[1,2,*]
[1]Lewis-Sigler Institute for Integrative Genomics
[2]Department of Molecular Biology
Princeton University
Princeton, New Jersey 08544

Summary

Background: Previous genetic evidence suggested that the *C. elegans* TGF-β Dauer pathway is responsible solely for the regulation of dauer formation, with no role in longevity regulation, whereas the insulin/IGF-1 signaling (IIS) pathway regulates both dauer formation and longevity.
Results: We have uncovered a significant longevity-regulating activity by the TGF-β Dauer pathway that is masked by an egg-laying (Egl) phenotype; mutants in the pathway display up to 2-fold increases in life span. The expression profiles of adult TGF-β mutants overlap significantly with IIS pathway profiles: Adult TGF-β mutants regulate the transcription of many DAF-16-regulated genes, including genes that regulate life span, the two pathways share enriched Gene Ontology categories, and a motif previously associated with DAF-16-regulated transcription (the DAE, or DAF-16-associated element) is overrepresented in the promoters of TGF-β regulated genes. The TGF-β Dauer pathway's regulation of longevity appears to be mediated at least in part through insulin interactions with the IIS pathway and the regulation of DAF-16 localization.
Conclusions: Together, our results suggest there are TGF-β-specific downstream targets and functions, but that the TGF-β and IIS pathways might be more tightly linked in the regulation of longevity than has been previously appreciated.

Introduction

In times of environmental stress, crowded conditions, and limited food, juvenile *C. elegans* develop into an alternative larval state called dauer that is highly stress resistant and long lived [1]. The decision to enter the dauer state is made during the first larval stage and is regulated by a complex branched pathway of more than 30 *daf* (dauer formation) genes [1]. Genetic epistasis tests suggested that components of one of the two canonical TGF-β signaling pathways (the TGF-β-like ligand DAF-7, the Type 1 and 2 receptors DAF-1 and DAF-4, and the downstream DAF-3 Smad and DAF-5 Sno/Ski) make up one branch of the dauer regulation pathway, whereas insulin/IGF-1 signaling (IIS) pathway genes, including the insulin receptor, DAF-2, and the FOXO transcription factor, DAF-16, occupy a separate downstream branch regulating both dauer formation and longevity [1–3]. The TGF-β dauer pathway has not been previously implicated in the regulation of longevity.

Transcriptional analyses have been carried out on wild-type dauer larvae [4], TGF-β mutant dauer larvae [5], and adult IIS pathway mutants [6–8]. However, the transcriptional profiles of TGF-β Dauer pathway mutant adults have not yet been examined; this is important for the temporal separation of developmental and adult targets. We hypothesized that the transcriptional targets of these pathways likely share dauer-specific targets [5, 8] but should diverge significantly in adults if longevity determination is a primary output solely of the IIS pathway.

Here, we have examined the transcriptional output from the adult TGF-β pathway and compared it to the profiles of TGF-β dauer animals and adult IIS pathway mutants. Surprisingly, the adult TGF-β profiles correlated well with the IIS pathway profile, suggesting that we should re-examine the longevity phenotype of the TGF-β mutants and the relationship between the TGF-β and insulin/IGF-1 signaling pathways.

Results

The Adult TGF-β Dauer and IIS Pathways Share Transcriptional Outputs and GO Terms

To identify the downstream targets of the TGF-β pathway in adulthood, we compared the dauer-constitutive mutants *daf-7(e1372)*, *daf-7(m62)*, and *daf-1(m40)* with dauer-defective mutants *daf-3(mgDf90)*, *daf-5(e1386)*, and *daf-7(e1372);daf-3(mgDf90)* double mutants at the permissive temperature, 20°C, on the first day of adulthood (see Figure 1 and the Experimental Procedures for hybridization details). Because *daf-3* and *daf-5* are epistatic to *daf-7* and *daf-1*, these comparisons should identify targets that act downstream of this linear pathway. We compared these profiles with expression data from Liu et al. [5] of dauer-stage *daf-7(e1372)*, *daf-8(e1393)*, and *daf-14(m77)* mutants versus wild-type L2/L3. Surprisingly, the Pearson correlation between the transcriptional outputs of the TGF-β dauer and adult pathways is low (0.004; Figure 1A), suggesting that the downstream targets of this pathway vary significantly from dauers to adults.

We used significance analysis of microarrays (SAM) [9], which provides an estimate of false discovery rate for multiple hypothesis testing, to identify genes consistently and significantly changed in TGF-β adults (Table S1 and Figure S2 in the Supplemental Data available online) and compared them with the Liu et al. dauer data [5]. As the Pearson correlation suggested, only 14% of the upregulated and 37% of the downregulated genes are shared between dauer and adult TGF-β pathway mutants. By contrast, the IIS pathway and the adult TGF-β pathway share significant similarity in transcriptional output, as demonstrated by a Pearson correlation of 0.35, almost 90-fold higher than the adult-dauer correlation (Figure 1B, Figure S1). We compared the

*Correspondence: ctmurphy@princeton.edu

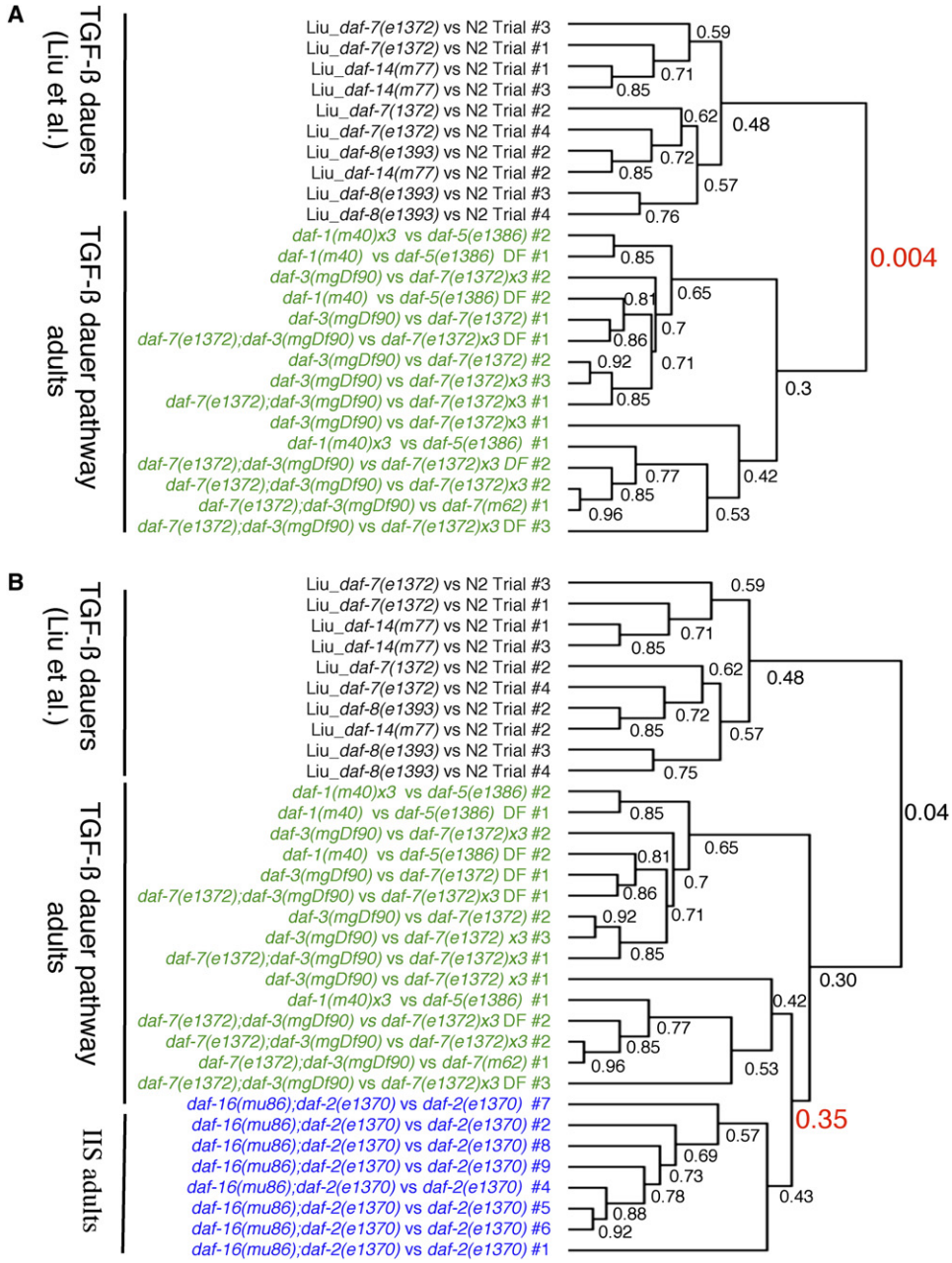


Figure 1. Hierarchical Clustering and Correlations between the TGF-β Dauer and Insulin/IGF-1 Signaling Pathways

TGF-β adult expression profiles are more similar to IIS adults than to TGF-β dauers, and adding the IIS pathway to the TGF-β adult clade increases its similarity to TGF-β dauers. "DF" indicates dye flip. (A) shows TGF-β dauer stage [5] and TGF-β adult profiles (Pearson correlation = 0.004), and (B) shows TGF-β adults and IIS adults (Pearson correlation = 0.35); the correlation between the TGF-β dauers and all adults increases 10-fold with IIS inclusion. Note that TGF-β adults and IIS adults Pearson correlations remained high with six IIS arrays (0.42, Figure S1), when only the adult profiles for the two pathways were compared (0.348) and when all of the TGF-β arrays are forced into a single clade and compared with the IIS clade (0.32).

SAM-determined significantly regulated genes in the IIS pathway [6] and found that 55% of the genes significantly upregulated by the IIS pathway and 66% of the downregulated genes are also regulated by the adult TGF-β pathway in the same direction (Table S2).

To compare the biological roles that the genes regulated by each of these pathways might play, we submitted the lists of significantly up- and downregulated genes from both the adult TGF-β SAM analysis and from the TGF-β dauer arrays [5] to DAVID [10] for Gene Ontology (GO) analysis. The TGF-β adults and TGF-β dauers' most-enriched GO categories were then compared (Figure 2; note that because GO terms are not independent, a statistical assessment of the degree of overlap is not possible; thus, we have graphically compared sets of enriched GO terms). Some categories are shared between the dauer and adult upregulated (Figure 2A, 9 of 37) and downregulated (Figure 2B, 14

of 47) sets. However, many of the top-enriched categories from the up- (28/37) and downregulated (33/47) gene sets are specific for either the adult or the dauer-stage TGF-β worms. The TGF-β dauer and adult IIS pathways share several GO categories (8/35 upregulated, 13/40 downregulated), including longevity-associated terms, perhaps reflecting the longevity of dauer animals.

Comparison of the GO categories most highly enriched by the IIS and adult TGF-β pathways reveals that several of the highest-scoring GO categories in both the up- and downregulated sets overlap: 16 of the 41 upregulated categories (Figure 2A) and 16 of the 38 downregulated categories (Figure 2B) are shared between the TGF-β adult and IIS profiles. Thus, by Pearson correlation of whole transcriptome, the percentage of significantly regulated genes, and GO category analysis, the adult TGF-β pathway appears to have much more in common with the adult IIS pathway than might have been expected from its reported phenotypes. More surprisingly, many of the GO categories that are known to function in longevity regulation (Figure 2A, asterisks) [6] were enriched in the TGF-β mutant adults.

The DAF-16-Associated Element Is Enriched in the Promoters of Adult TGF-β Dauer Pathway Targets

We then identified clusters [11] of genes that are up- or downregulated and shared or distinct across the strains (Figure 3, Table S3), similar to the approach previously used to find longevity genes regulated by *daf-2* and *daf-16* [6]. These comparisons allowed us to identify TGF-β-regulated genes that are specific to and shared in dauer and adulthood. The transcriptional profiles of adult TGF-β and IIS pathway mutants show that these pathways share many targets in adulthood (Figure 3, clusters 3 and 5). Strikingly, many of the *dod* genes (downstream of DAF-16) responsible for *daf-2* insulin receptor mutants' long life [6] are highly regulated by the TGF-β pathway, in the same direction as in the IIS pathway [6]. Specifically, *sod-3*, *mtl-1*, *dod-3*, *dod-12/acdh-1*, *dod-11/sodh-1*, *lys-7*, *dao-3*, *gei-7*, *dod-4/aqp-1*, *dod-9/acs-17*, *ges-1*, *dod-7/asah-1*, *gcp-1*, *dod-6*, *ins-18*, *fat-7*, *mdl-1*, *spp-1*, *dod-24*, *dod-22*, and *dod-18*, which had all been previously identified as life-span-regulating *dod* genes [6], were notable because of their strong expression in TGF-β adults (Figure S3).

We submitted the promoter sequences from the cluster gene lists to two complementary motif-finding algorithms, BioProspector [12] and Weeder [13], to identify overrepresented promoter sequences (Figure 3). The yeast-one-hybrid-identified DAF-3 binding sequence, GTCTG, which could direct pharyngeal gene expression during larval development [14], was not apparent in any of the high-scoring motifs. By contrast, the DAF-16-associated element (DAE) (CTTATCA), a GATA-like motif that was first identified as an overrepresented sequence in the promoters of genes regulated by DAF-16 [6], and variations of the DAE were the most common sequences in the promoters of these genes. Although the presence of the DAE in the promoters of genes regulated by both the IIS and adult TGF-β pathway could be explained by the presence of the IIS genes, the DAE was also strongly associated with Cluster 1 (Figure 3), which is regulated specifically by the TGF-β pathway.

TGF-β Dauer Pathway Mutants Regulate Longevity

The striking regulation of insulin transcriptional targets previously demonstrated to regulate life span [6] by adult TGF-β mutants (Figure S3) suggested that the TGF-β pathway might also regulate life span, despite previously published longevity measurements [2, 3]. To test this hypothesis, we measured the life spans of ten different TGF-β Dauer pathway mutant alleles. Because these mutants are known to have severe egg-laying (Egl) defects [3, 15], we were concerned that matricide (progeny hatching within the mother, or "bagging") might cause premature death. In fact, we found that *daf-7* animals display high rates of bagging compared with the wild-type (Figure 4A). Therefore, we used the chemical 5-fluorodeoxyuridine (FUdR), an inhibitor of DNA synthesis, to prevent progeny development [16]. FUdR treatment itself has minimal effects on wild-type life span [16] (Table 1, Tables S4–S6).

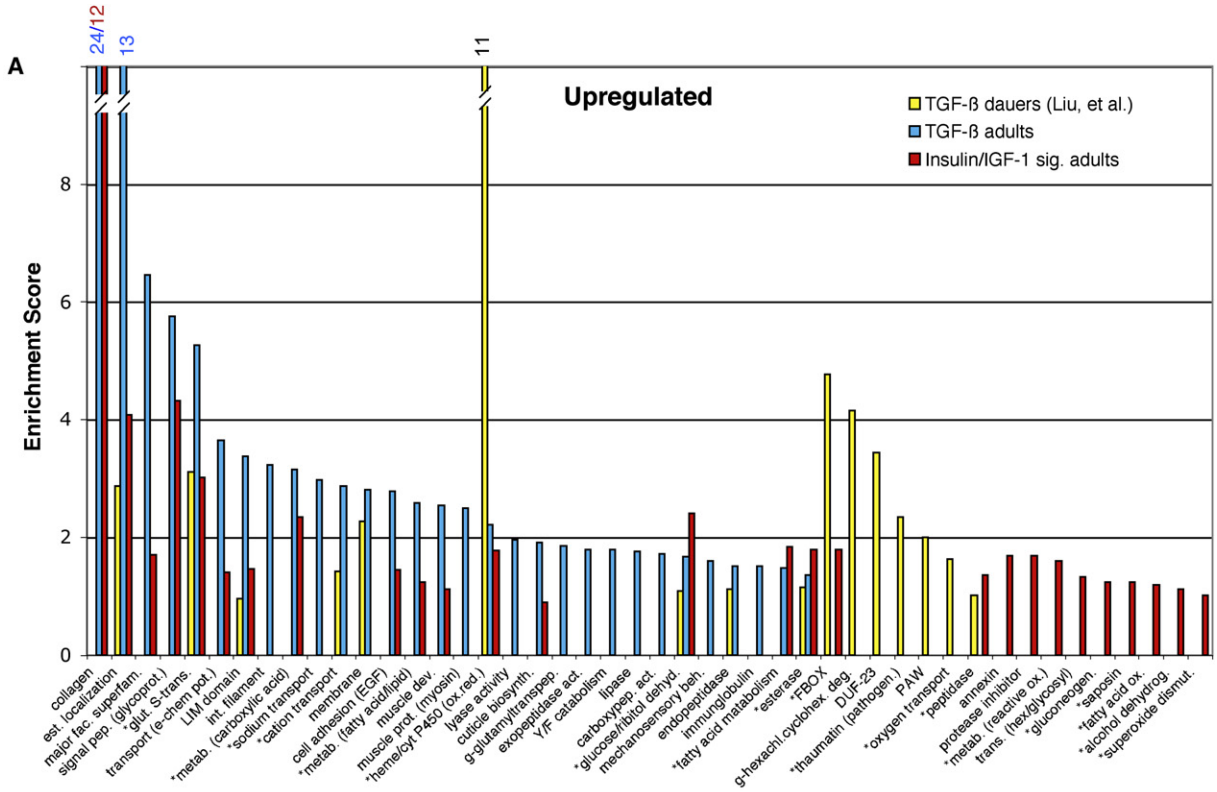
Surprisingly, most mutants in the Dauer pathway extended life span substantially and consistently: We observed a significant increase in life span in *daf-7*, *daf-1*, *daf-4*, *daf-8*, and *daf-14* mutants relative to wild-type worms under the same conditions (Figures 4B–4D, Table 1). In fact, *daf-4(e1364)* mutants lived more than twice as long as the wild-type in one trial (Figure 4C, Table 1). Meanwhile, negative regulators of the dauer pathway (*bra-1*, *daf-5*, and *daf-3*) displayed significant shortening of life span relative to the wild-type (Figures 4B and 4D, Table 1). The long life span of *daf-7* mutants was suppressed by *daf-3* mutations (Figure 4E, Table S4), similar to *daf-3*'s suppression of *daf-7*-mediated dauer formation.

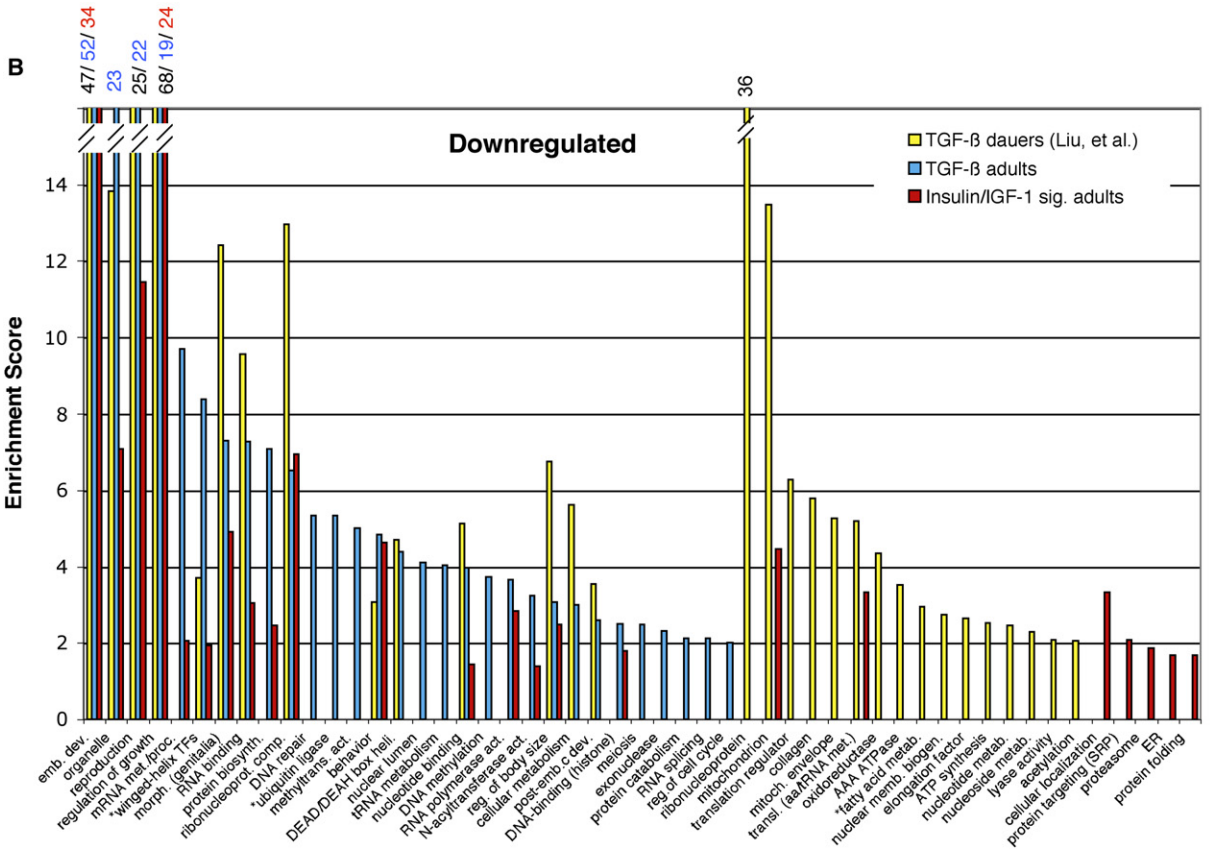
Regulation of Life Span by the TGF-β Dauer Pathway Requires DAF-16 Activity

Previous studies suggested that the TGF-β and IIS pathways function independently [2, 3], although *daf-16* mutations have been reported to partially suppress dauer formation of TGF-β mutants [3]. To investigate the interaction between the two pathways in longevity regulation, we measured the life span of the *daf-16(mu86);daf-7(e1372)* double mutant. We found that loss of *daf-16* activity completely suppressed the longevity phenotype of *daf-7(-)* mutants or RNAi (RNA interference) (Figure 4F, Table S5). Furthermore, the loss of *daf-16* suppressed the slight thermotolerance displayed by *daf-7* (Figure 4H). By contrast, the loss of *daf-3* was not able to suppress the life span extension caused by *daf-2* mutations or RNAi (Figure 4G, Table S6), suggesting unidirectional communication from the TGF-β Dauer pathway to the IIS/FOXO pathway in longevity regulation.

The TGF-β Dauer Pathway Acts in Adulthood to Regulate Longevity

Reduction of IIS pathway signaling during adulthood is sufficient to increase longevity [17], but the dauer decision is made in the L1 larval stage [1]. To determine when TGF-β pathway activity affects life span, we carried out a series of temporal temperature-shift and double-stranded RNA interference experiments. When we raised *daf-7(e1372)ts* or *daf-7(m62)ts* animals at 20°C and then shifted them to 15°C as young adults, no life span extension was observed (Figure 5B). By contrast, when these worms were raised at 15°C and then shifted


A
Upregulated
TGF-ß dauers (Liu, et al.)
TGF-ß adults
Insulin/IGF-1 sig. adults
Enrichment Score
24/12
13
11
0
2
4
6
8
collagen
est. localization
major fac. superfam.
signal pep. (glycoprot.)
*glut. S-trans.
transport (e-chem pot.)
LIM domain
int. filament
*metab. (carboxylic acid)
*sodium transport
*cation transport
membrane
cell adhesion (EGF)
*metab. (fatty acid/lipid)
muscle dev.
muscle prot. (myosin)
*heme/cyt P450 (ox.red.)
lyase activity
cuticle biosynth.
g-glutamyltranspep.
exopeptidase act.
Y/F catabolism
lipase
carboxypep. act.
*glucose/hitol dehyd.
mechanosensory beh.
endopeptidase
immunglobulin
*fatty acid metabolism
*esterase
*FBOX
g-hexachl.cyclohex. deg.
DUF-23
*thaumatin (pathogen.)
PAW
*oxygen transport
*peptidase
annexin
protease inhibitor
*metab. (reactive ox.)
trans. (hex/glycosyl)
*gluconeogen.
*saposin
*fatty acid ox.
*alcohol dehydrog.
*superoxide dismut.


B
Downregulated
TGF-ß dauers (Liu, et al.)
TGF-ß adults
Insulin/IGF-1 sig. adults
Enrichment Score
47/ 52/ 34
23
25/ 22
68/ 19/ 24
36
0
2
4
6
8
10
12
14
emb. dev.
organelle
reproduction
regulation of growth
mRNA met./proc.
*winged-helix TFs
morph. (genitalia)
RNA binding
protein biosynth.
ribonucleoprot. comp.
DNA repair
*ubiquitin ligase
methyltrans. act.
behavior
DEAD/DEAH box heli.
nuclear lumen
tRNA metabolism
nucleotide binding
DNA methylation
RNA polymerase act.
N-acyltransferase act.
reg. of body size
cellular metabolism
post-emb c dev.
DNA-binding (histone)
meiosis
exonuclease
protein catabolism
RNA splicing
reg of cell cycle
ribonucleoprotein
mitochondrion
translation regulator
collagen
mitoch. envelope
transl. (aa/tRNA met.)
oxidoreductase
AAA ATPase
*fatty acid metab.
nuclear memb. biogen.
elongation factor
ATP synthesis
nucleotide metab.
nucleoside metab.
lyase activity
acetylation
cellular localization
protein targeting (SRP)
proteasome
ER
protein folding

to 20°C as L4/young adults, life span was increased significantly ($p < 0.0001$) (Figure 5C). Furthermore, when wild-type worms were treated with *daf-7* RNAi only in adulthood, life span was also increased ($p < 0.0001$) (Figure 5D). Thus, *daf-7* longevity activity is separable from the dauer decision and formation *daf-7* activity in larval stages, and, like the insulin pathway, the TGF-β pathway acts during adulthood rather than in larval stages to regulate longevity.

The TGF-β Dauer Pathway Regulates DAF-16 Localization and DAF-16 Target-Gene Transcription

The suppression of *daf-7* mutants' extended life span by *daf-16* mutations suggests that the TGF-β Dauer pathway regulates IIS-pathway activity in adults. When *daf-2* insulin receptor signaling is abrogated, DAF-16/FOXO becomes nuclearly localized [18, 19], and mutations in *daf-18,* the PTEN phosphatase that opposes insulin and PI-3-kinase signaling, promote the cytoplasmic retention of DAF-16 [18]. *daf-7(m62)* dauer animals were previously shown to nuclearly localize DAF-16 during the L2d predauer state [20]. We found that DAF-16::GFP was excluded from nuclei in many adult *daf-3* mutants (Figures 6A–6C) and with *daf-3* RNAi, similar to *daf-18* mutants [18]. *daf-7(e1372);daf-16::gfp* adults were also heterogeneous, but generally shifted DAF-16::GFP localization from diffuse to nuclearly localized (Figures 6A–6C). These results suggest that the TGF-β Dauer pathway might act through an insulin-signaling-like regulation of DAF-16 localization.

sod-3 (superoxide dismutase) is a direct transcriptional target of DAF-16 [21, 22], and the expression of a *Psod-3::gfp* reporter is increased broadly in *daf-2* mutants [23]. Our microarray results suggested that like the IIS pathway, TGF-β Dauer mutants regulate the transcription of *sod-3* (Figure S3). We examined *daf-7(e1372);Psod-3::gfp* mutants and found that fluorescence was increased in intestines, hypodermis, and cuticle, although this pattern was weaker and more heterogeneous than in *daf-2* mutants. *Psod-3::gfp* expression was not noticeably altered below wild-type levels in *daf-3(mgDf90)* mutants (Figure 6D), where expression is already low and restricted to the pharynx and tail. *daf-3* RNAi did not have a notable effect on the high *Psod-3::gfp* expression in *daf-2* mutants, correlating with its lack of *daf-2* longevity suppression. Together these results suggest that the *daf-2/daf-16* pathway acts downstream of *daf-7* and *daf-3* (Figure 6E).

Discussion

The TGF-β Pathway Regulates Longevity through Insulin/IGF-1 Signaling

Our transcriptional analyses of adult TGF-β mutants have identified a number of adult transcriptional targets, functional categories, and regulatory motifs [5, 6] that are shared between the TGF-β and IIS pathways, including many *daf-2/daf-16* target genes demonstrated to regulate longevity [6]. These transcriptional results directly suggested the possibility that the TGF-β pathway might regulate life span. Supportive of this, we found that TGF-β mutants are indeed long lived, that the TGF-β pathway acts during adulthood to regulate life span, and that this regulation depends on the FOXO transcription factor, DAF-16. Together, our results suggest that the TGF-β and insulin/IGF-1 pathways use similar transcriptionally regulated mechanisms to survive, and that these pathways are more closely linked than has been previously appreciated.

One interesting difference between dauer and longevity regulation is that loss of *daf-16* is only able to partially suppress *daf-7* dauer formation [3], but it completely suppresses the life span extension of *daf-7*, which is weaker than *daf-2*'s longevity effect. This might indicate a greater role by DAF-3 in dauer formation and a more specific role for DAF-16 in longevity regulation. Nevertheless, DAF-16 might play at least a partial role in the regulation of *daf-7* dauer transcriptional targets and the partial suppression of dauer formation.

Lee et al. also noted *daf-7* regulation of DAF-16::GFP in L2d animals [20] and suggested that an insulin might mediate the interaction between the TGF-β and IIS/FOXO pathways, and Liu et al. found several insulins regulated by TGF-β mutants in dauer [5]. The regulation of an insulin-like peptide gene or genes by DAF-3 in adulthood (Figure 6E, Figure S4) would provide a powerful mechanism for the activation of the IIS pathway and the coordination of the two pathways, similar to the proposed coordination of the IIS pathway through *ins-7* signaling [6]. An insulin-based mechanism might allow the coordination of the TGF-β and IIS pathways throughout the animal, reinforcing the dauer decision during larval stages and longevity regulation in adults.

Matricide Masks Longevity Phenotypes of TGF-β Dauer Mutants

Our finding that the TGF-β dauer pathway can regulate life span through its interaction with the insulin/IGF-1 pathway suggests that dauer survival and longevity regulation might be even more closely linked than earlier genetic studies had indicated. Although it is surprising that such significant life span increases had not been previously reported for any of the mutants in this pathway, it should be noted that some studies had not prevented progeny production to reduce matricide. In other studies, *fer* mutations were used to confer partial sterility (because of the loss of sperm progeny production); however, we have observed that *fer* mutations might also have a slightly deleterious effect on life span in TGF-β mutant backgrounds (Figure S5). Previous studies also measured life span at 25.5°C, which might have caused more severe deleterious effects, either

Figure 2. Gene Ontology Analysis of TGF-β Targets in Dauer and Adults

SAM-determined significantly changed genes in TGF-β dauers [5], TGF-β dauer pathway adults (Table S1), and IIS mutants (Table S2) were submitted for GO analysis and ordered by enrichment score in the TGF-β adult sets. (A) shows the GO categories of genes upregulated by TGF-β dauers (1381 genes), TGF-β adults (2181 genes), and IIS mutants (1390); (B) shows the GO categories of genes downregulated by TGF-β dauers (2725 genes), TGF-β adults (top 3000 genes), and IIS mutants (1054 genes). Asterisks indicate GO categories that are known to function in longevity regulation.

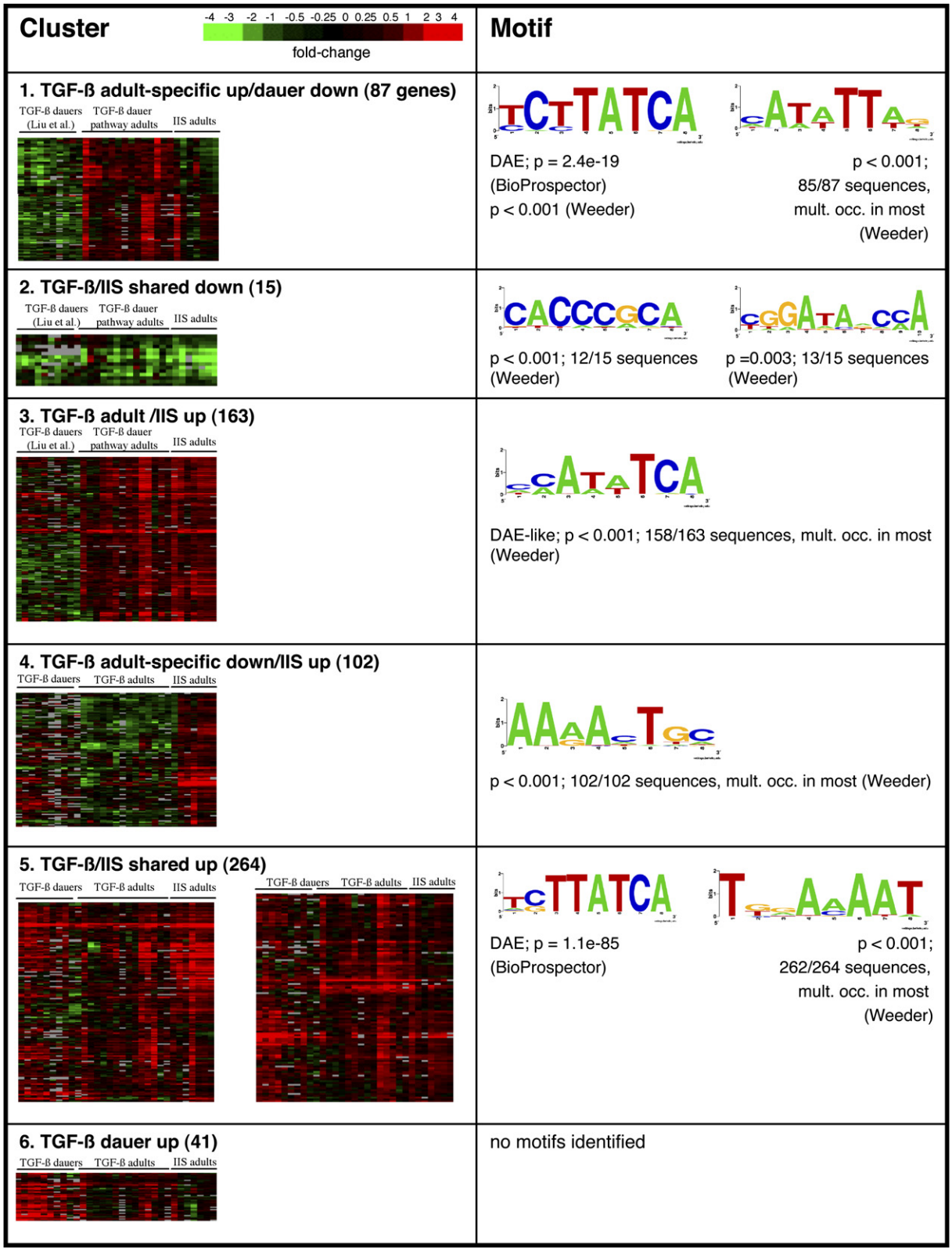


Figure 3. Transcriptional Targets of TGF-β Dauer Stage, TGF-β Adults, and IIS Adults with WebLogos of Associated Motifs

Arrays were hierarchically clustered, and 1.5 kb of promoter sequences from the genes in each cluster were submitted to BioProspector [12] and Weeder [12] so that overrepresented motifs could be identified; high-scoring motifs are depicted by WebLogo [31]. The DAF-16-associated element (DAE) and DAE-like motifs were overrepresented in the promoters of several gene clusters. "1" shows TGF-β adult-specific up- and TGF-β dauer-downregulated targets, "2" shows TGF-β/IIS shared downregulated targets, "3" shows TGF-β adult/IIS shared upregulated targets, "4" shows TGF-β adult-specific down/IIS upregulated targets, "5" shows TGF-β/IIS shared upregulated targets, and "6" shows TGF-β dauer stage upregulated targets.

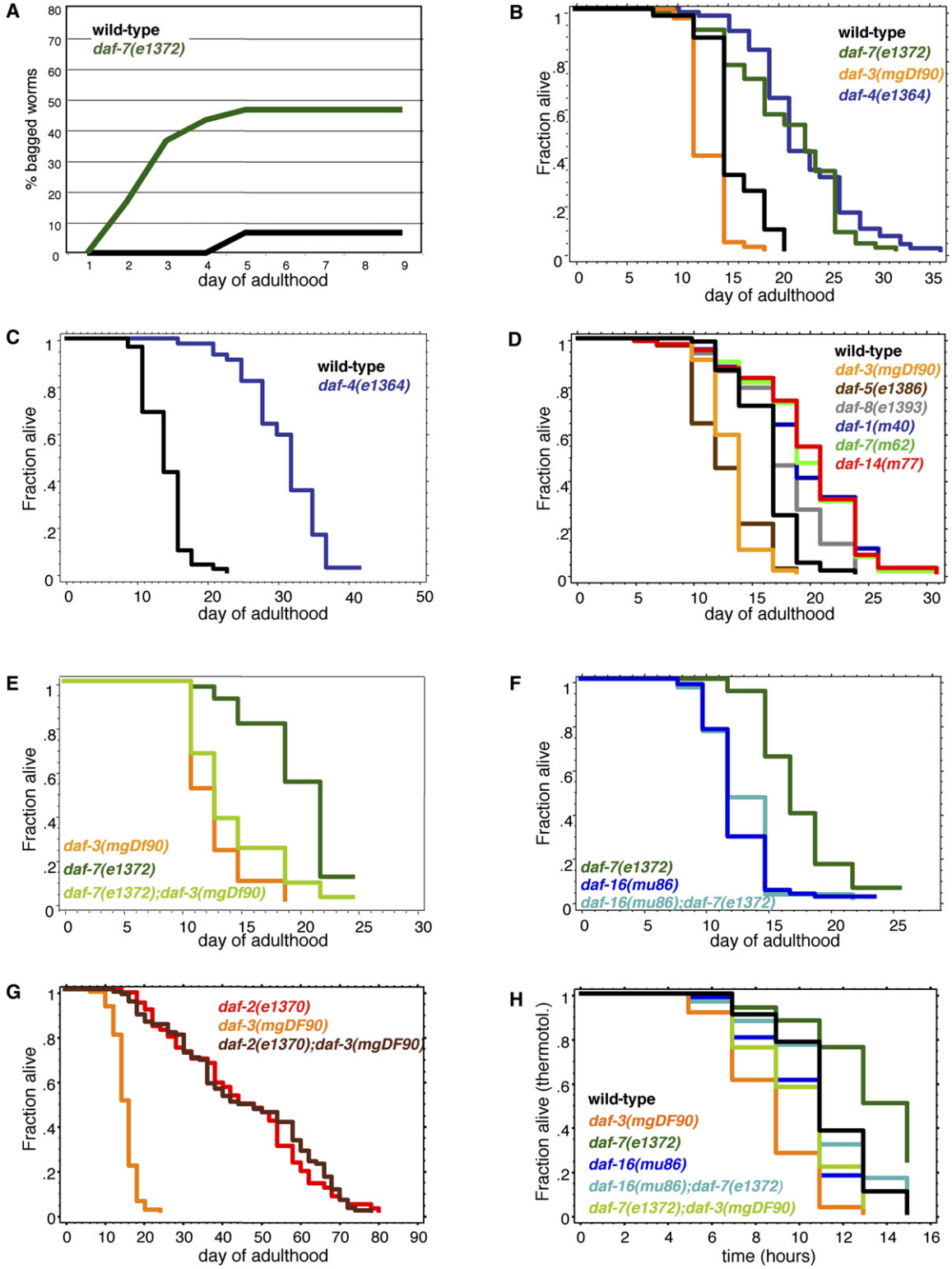


Figure 4. Longevity and Thermotolerance Are Regulated by TGF-β Dauer Pathway Signaling in a DAF-3- and DAF-16-Dependent Manner

See Figure S6 in the Supplemental Data for an expanded legend, including life-span data.

(A) *daf-7(e1372)* matricide rates (20°C, no FUdR).

(B–G) Life spans of hermaphrodites treated with 50 μM FUdR during early adulthood to prevent progeny development. *Daf-c* mutants (*daf-7*, *daf-4*, *daf-8*, *daf-1*, and *daf-14)* are long lived, whereas *Daf-d* mutants (*daf-3* and *daf-5)* are short lived (B, C, and D). *daf-7(e1372)* mutant life span extension is dependent on both *daf-3* (E) (eight replicates, Table S4) and *daf-16* activity (F) (six replicates, Table S5). *daf-2* longevity is independent of *daf-3* (G) (seven replicates, Table S6).

(H) TGF-β mutants are thermotolerant (35°C) in a *daf-3*-, *daf-16*-dependent manner. (*daf-2(e1370)* was very thermotolerant in this assay, with a mean of 21.2 hr, and 100% were alive through the course of the assay shown.)

Table 1. Life Spans of TGF-β Dauer Pathway Mutants

Genotype	Mean Life Span	Standard Error	p Value	Percent Change
Experiment #1				
wild type	15.8	0.4	–	–
daf-7(e1372)	21.1	0.7	<0.0001	+33%
daf-4(e1364)	22.1	0.6	<0.0001	+40%
daf-3(mgDf90)	13.2	0.3	<0.0001	−16%
Experiment #2				
wild type	15.7	0.4	–	–
daf-7(e1372)	17.8	0.8	0.15	+13%
daf-3(mgDf90)	13.2	0.3	<0.0001	−16%
Experiment #3				
wild-type	14.4	0.3	–	–
daf-1(m40)	21.0	0.6	<0.0001	+46%
bra-1(nk1)	10.8	0.2	<0.0001	−25%
daf-3(e1376)	14.4	0.2	0.76	0%
Experiment #4				
wild type	13.5	0.3	–	–
daf-7(e1372)	18.8	0.8	<0.0001	+39%
daf-7(m62)	16.3	0.5	<0.0001	+21%
daf-8(e1393)	17.7	0.5	<0.0001	+31%
daf-14(m77)	19.4	0.5	<0.0001	+44%
daf-3(mgDf90)	12.0	0.2	<0.0001	−11%
Experiment #5				
wild type	14.2	0.4	–	–
daf-7(e1372)	18.9	0.7	<0.0001	+33%
daf-7(m62)	18.0	0.5	<0.0001	+27%
daf-4(e1364)	31.0	0.8	<0.0001	+120%
Experiment #6				
wild type	16.4	0.2	–	–
daf-7(e1372)	16.5	0.4	0.45	0%
daf-7(m62)	19.7	0.5	<0.0001	+20%
daf-1(m40)	19.5	0.5	<0.0001	+19%
daf-8(e1393)	17.8	0.4	0.3	+9%
daf-14(m77)	19.9	0.6	<0.0001	+21%
bra-1(nk1)	15.4	0.3	0.2	−6%
daf-5(e1386)	13.3	0.4	<0.0001	−19%
daf-3(e1376)	15.5	0.4	0.03	−6%
daf-3(mgDf90)	13.3	0.2	<0.0001	−19%
Experiment #7				
wild type	16.8	0.4	–	–
daf-7(e1372), 3x outcrossed	20.8	1.7	<0.0001	+24%
daf-7(m62), 3x outcrossed	22.2	0.6	<0.0001	+29%
daf-14(m77), 3x outcrossed	20.5	0.4	<0.0001	+24%
daf-1(m40), 3x outcrossed	19.9	0.4	<0.0001	+18%

Life-span experiments were carried out at 20°C on 50 μM FUdR; n > 60 for each sample.

from TGF-β dauer mutant or *fer* pleiotropies, than the temperature we have used here (20°C). Finally, complete deletion of bagged animals from the entire life span, rather than standard survival analysis censoring (in which the animal contributes to the "live" and total population until the point at which it is removed), was used in the calculation of life span in one study [2], and the second did not censor for bagging events after day 7 [3], when we continue to observe matricide (Figure 4A). It is also not surprising that RNAi longevity screens did not pick up these genes, despite the use of sterile strains [24–27], because the *daf-7* RNAi clone appears to act more weakly than does the mutant allele and might not have met the maximum longevity requirement of these screens (Figure 5). Interestingly, another study did identify *daf-7* and *daf-1* in tests of L1 starvation-induced increased longevity [28], and noted their increased thermotolerance as well, but discounted them for further adult longevity tests on the basis of previously published life span reports. In general, it is possible that matricide contributes to deaths more frequently than is currently appreciated, and the suppression of matricide might reveal additional longevity regulators.

Implications for Higher Organisms

The conservation of TGF-β and insulin signaling pathways between *C. elegans* and mammals is significant. It is possible that more interaction between these pathways than has been previously suspected might also exist in higher organisms, and might affect the survival of

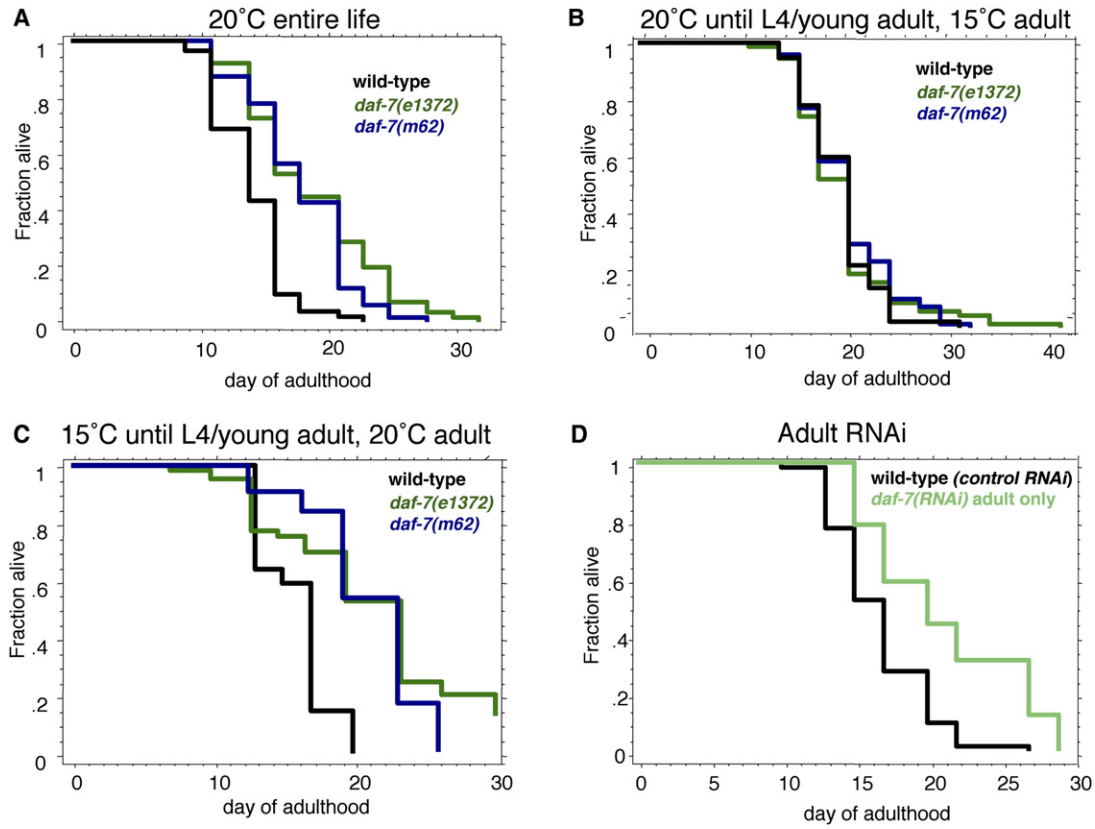


Figure 5. Temporal Analysis of *daf-7* Longevity Regulation

(A) Life span of wild-type (14.2 ± 0.4), *daf-7(e1372)* (18.9 ± 0.7, p < 0.0001), and *daf-7(m62)* (18.0 ± 0.5, p < 0.0001) worms at 20°C their entire life.
(B) Life span of wild-type (20.1 ± 0.5), *daf-7(e1372)* (20.3 ± 0.6, p = 0.9), and *daf-7(m62)* (20.8 ± 0.5, p = 0.9) worms raised at 20°C until L4/young adulthood then shifted to 15°C.
(C) Life span of wild-type (15.9 ± 0.5), *daf-7(e1372)* (21.6 ± 0.9, p < 0.0001), and *daf-7(m62)* (20.8 ± 0.5, p < 0.0001) worms raised at 15°C until L4/young adulthood, then shifted to 20°C.
(D) Life span of wild-type worms treated with vector control (16.7 ± 0.4) or *daf-7* RNAi during adulthood only (21.1 ± 1.1, p < 0.0001).

specific tissues and, ultimately, the longevity of these animals. The work presented here suggests that the role of the TGF-β signaling in life span regulation and the interactions between the TGF-β and insulin/IGF-1 signaling pathways should also be investigated in higher organisms.

Experimental Procedures

C. elegans Genetics

All strains were cultured at 20°C, unless otherwise noted, with standard methods [29]. Strain and allele information is listed in the Supplemental Data.

RNA Preparation and Microarray Hybridization

Standard RNA purification, cRNA generation, labeling, and hybridization on Agilent 4 × 44K *C. elegans* arrays were performed (Supplemental Data). Fifteen replicates of TGF-β adults were hybridized [4 × *daf-1(m40) v daf-5(e1386)* (two dye flipped), 10 × *daf-7(e1372) v daf-3(mgDf90)* or *daf-7(e1372);daf-3(mgDf90)* (four dye flipped), and 1 × *daf-7(m62) v daf-7(e1372);daf-3(mgDf90)*]. Nine replicates of *daf-2(e1370) v daf-16(mu86);daf-2(e1370)* (four dye flips) were hybridized.

Microarray Analysis

Data was loaded onto the Princeton University MicroArray database (PUMA [http://puma.princeton.edu]) and filtered for array and spot quality, and replicate spots were collapsed to an average value. One-class analysis in SAM [9] was performed with no expression level cutoff. Hierarchical clustering by gene and arrays of $\log_2$ expression rations was done after genes that were present in 80% of the arrays were filtered (uncentered correlation, average linking) in Cluster and displayed in TreeView [11].

Gene Ontology Analysis

Up- and downregulated genes identified in the SAM analyses were converted to SwissProt ID and TrEMBLE ID's in WormBase and submitted to DAVID for conversion to DAVID ID's for functional annotation clustering (>70% converted). Molecular and/or biological functional annotation was assigned to the upregulated and downregulated genes with the default *C. elegans* Gene Ontology criteria in DAVID (http://david.abcc.ncifcrf.gov/), and the enrichment score (the −log of the Fisher's exact test p value) was determined [10]. All categories with an enrichment score greater than 1 or the top 30 GO categories are shown (Figure 2).

Motif Analysis

Upstream sequences (1.5 kb, WormBase Release WS170) were submitted to two complementary algorithms, BioProspector [12] (a Gibbs sampling-based algorithm) and Weeder [13] (a consensus-based algorithm), so that overrepresented sequences could be identified. p values for BioProspector motifs were generated by the performance of 35 Monte Carlo simulations so that the null motif score distribution could be estimated [12]; Weeder's p value calculator was used for motif significance. Motifs were displayed in WebLogo [30].

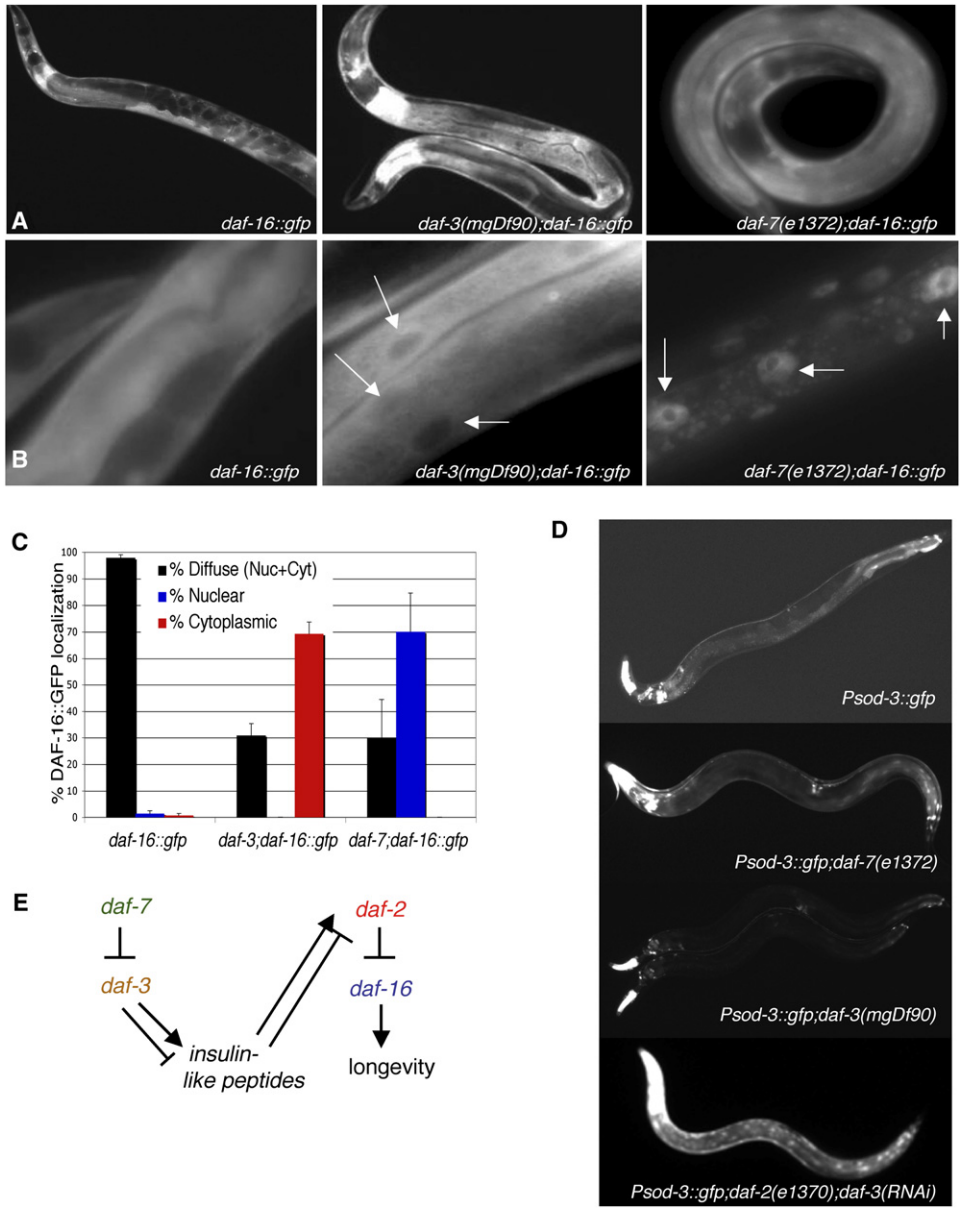


Figure 6. The TGF-β Dauer Pathway Regulates DAF-16 Localization and *sod-3* Transcription

(A and B) Images of DAF-16::GFP animals at 100× (A) and 400× (B). DAF-16::GFP remains diffuse in a wild-type background, but it is excluded from nuclei in many *daf-3* animals and is partially nuclearly localized in many *daf-7* mutants.

(C) Ratios of animals with diffuse (black), nuclearly localized (blue), and cytoplasmic (red) DAF-16::GFP in wild-type *daf-3(mgDf90)* and *daf-7(e1372)* mutant backgrounds ± SEP.

(D) *Psod-3::gfp*, *Psod-3::gfp;daf-7(e1372)*, *Psod-3::gfp;daf-3(mgDf90)*, and *daf-2(e1370);Psod-3::gfp;daf-3(RNAi)* animals.

(E) Model of TGF-β Dauer and IIS pathway interactions regulating longevity.

Survival Analysis

Standard Kaplan-Meier survival analysis [31] (first day of adulthood as t = 0, 20°C unless otherwise indicated, n > 60, 50 μM FUdR) was performed (Supplemental Data).

RNAi

Bacterial feeding RNAi experiments were carried out as described previously [17, 32] after verification by polymerase chain reaction (PCR) and sequencing, on 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG).

Thermotolerance Assay

n > 60 day 1 adult worms (treated with FUdR from L4) were shifted from 20°C to 35°C at t = 0, and time points were taken every 2 hr after 3 hr of incubation at 35°C; standard Kaplan-Meier survival analysis was performed.

DAF-16::GFP Localization Assay

Day 1 adult worms were scored (three assays) for nuclear, cytoplasmic, or diffuse green fluorescent protein (GFP) localization, as in [33], and plotted as a percentage of the total ± the standard error of the proportion (SEP).

Supplemental Data

Experimental Procedures, expanded figure legend, five figures, and six tables are available at http://www.current-biology.com/cgi/content/full/17/19/1635/DC1/.

Acknowledgments

We thank the Center for *C. elegans* Genetics for strains, John Matese and Laurie Kramer for assistance with PUMA, Hani Goodarzi, Max Jan, and Kieran Dilks for technical assistance, Zemer Gitai for comments on the manuscript, and Chad Myers for discussion. W.M.S. and C.T.M. designed the experiments, W.M.S., S.L., J.A., and C.T.M. carried out the experiments, J.L. performed motif analyses, and W.M.S., S.L, J.L., and C.T.M. wrote the manuscript. W.M.S. is a fellow of the New Jersey Commission on Cancer Research. C.T.M. is an Alfred P. Sloan Fellow and a Pew Scholar in the Biomedical Sciences.

Received: June 21, 2007
Revised: August 8, 2007
Accepted: August 16, 2007
Published online: September 27, 2007

References

1. Riddle, D.L., and Albert, P.S. (1997). Genetic and environmental regulation of dauer larva development. In *C. elegans* II, D.L. Riddle, T. Blumenthal, B.J. Meyer, and J.R. Priess, eds. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), pp. 739–768.
2. Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. Nature *366*, 461–464.
3. Larsen, P.L., Albert, P.S., and Riddle, D.L. (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics *139*, 1567–1583.
4. Wang, J., and Kim, S.K. (2003). Global analysis of dauer gene expression in *Caenorhabditis elegans*. Development *130*, 1621–1634.
5. Liu, T., Zimmerman, K.K., and Patterson, G.I. (2004). Regulation of signaling genes by TGFbeta during entry into dauer diapause in C. elegans. BMC Dev. Biol. *4*, 11.
6. Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature *424*, 277–283.
7. McElwee, J., Bubb, K., and Thomas, J.H. (2003). Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. Aging Cell *2*, 111–121.
8. McElwee, J.J., Schuster, E., Blanc, E., Thomas, J.H., and Gems, D. (2004). Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. J. Biol. Chem. *279*, 44533–44543.
9. Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA *98*, 5116–5121.
10. Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., and Lempicki, R.A. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. *4*, 3.
11. Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*, 14863–14868.
12. Liu, X., Brutlag, D.L., and Liu, J.S. (2001). BioProspector: Discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes. Pacific Symposium on Biocomputing, 127–138.
13. Pavesi, G., Mereghetti, P., Mauri, G., and Pesole, G. (2004). Weeder Web: Discovery of transcription factor binding sites in a set of sequences from co-regulated genes. Nucleic Acids Res. *32*, W199–W203.
14. Thatcher, J.D., Haun, C., and Okkema, P.G. (1999). The DAF-3 Smad binds DNA and represses gene expression in the Caenorhabditis elegans pharynx. Development *126*, 97–107.
15. Trent, C., Tsuing, N., and Horvitz, H.R. (1983). Egg-laying defective mutants of the nematode Caenorhabditis elegans. Genetics *104*, 619–647.
16. Gandhi, S., Santelli, J., Mitchell, D.H., Stiles, J.W., and Sanadi, D.R. (1980). A simple method for maintaining large, aging populations of Caenorhabditis elegans. Mech. Ageing Dev. *12*, 137–150.
17. Dillin, A., Crawford, D.K., and Kenyon, C. (2002). Timing requirements for insulin/IGF-1 signaling in *C. elegans*. Science *298*, 830–834.
18. Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. *28*, 139–145.
19. Henderson, S.T., and Johnson, T.E. (2001). *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr. Biol. *11*, 1975–1980.
20. Lee, R.Y., Hench, J., and Ruvkun, G. (2001). Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway. Curr. Biol. *11*, 1950–1957.
21. Honda, Y., and Honda, S. (1999). The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. FASEB J. *13*, 1385–1393.
22. Oh, S.W., Mukhopadhyay, A., Dixit, B.L., Raha, T., Green, M.R., and Tissenbaum, H.A. (2005). Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. Nat. Genet. *38*, 251–257.
23. Libina, N., Berman, J.R., and Kenyon, C. (2003). Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell *115*, 489–502.
24. Dillin, A., Hsu, A.L., Arantes-Oliveira, N., Lehrer-Graiwer, J., Hsin, H., Fraser, A.G., Kamath, R.S., Ahringer, J., and Kenyon, C. (2002). Rates of behavior and aging specified by mitochondrial function during development. Science *298*, 2398–2401.
25. Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G., and Lee, S.S. (2005). A systematic RNAi screen for longevity genes in C. elegans. Genes Dev. *19*, 1544–1555.
26. Hansen, M., Hsu, A.L., Dillin, A., and Kenyon, C. (2005). New Genes Tied to Endocrine, Metabolic, and Dietary Regulation of Lifespan from a Caenorhabditis elegans Genomic RNAi Screen. PLoS Genet *1*, e17.
27. Lee, S.S., Lee, R.Y.N., Fraser, A.G., Kamath, R.S., Ahringer, J., and Ruvkun, G. (2002). A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. Nature *33*, 40–48.
28. Munoz, M.J., and Riddle, D.L. (2003). Positive selection of Caenorhabditis elegans mutants with increased stress resistance and longevity. Genetics *163*, 171–180.
29. Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.
30. Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004). WebLogo: A sequence logo generator. Genome Res. *14*, 1188–1190.
31. Lawless, J.F. (1982). Models and Methods for Lifetime Data (New York: Wiley).
32. Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature *408*, 325–330.
33. Hertweck, M., Gobel, C., and Baumeister, R. (2004). C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. Dev. Cell *6*, 577–588.