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Direct and Indirect Gene Regulation by a Life-Extending FOXO Protein in *C. elegans*: Roles for GATA Factors and Lipid Gene Regulators

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SUMMARY

In long-lived *C. elegans* insulin/IGF-1 pathway mutants, the life-extending FOXO transcription factor DAF-16 is present throughout the animal, but we find that its activity in a single tissue can delay the aging of other tissues and extend the animal's life span. To better understand the topography of DAF-16 action among the tissues, we analyzed a collection of DAF-16-regulated genes. DAF-16 regulated most of these genes in a cell-autonomous fashion, often using tissue-specific GATA factors to direct their expression to specific tissues. DAF-16 could also act cell nonautonomously to influence gene expression. DAF-16 affected gene expression in other cells, at least in part, via the lipid-gene regulator MDT-15. DAF-16, and probably MDT-15, could act cell nonautonomously in the endoderm to ameliorate the paralysis caused by expressing Alzheimer's Aβ protein in muscles. These findings suggest that MDT-15-dependent intercellular signals, possibly lipid signals, can help to coordinate tissue physiology, enhance proteostasis, and extend life in response to DAF-16/FOXO activity.

INTRODUCTION

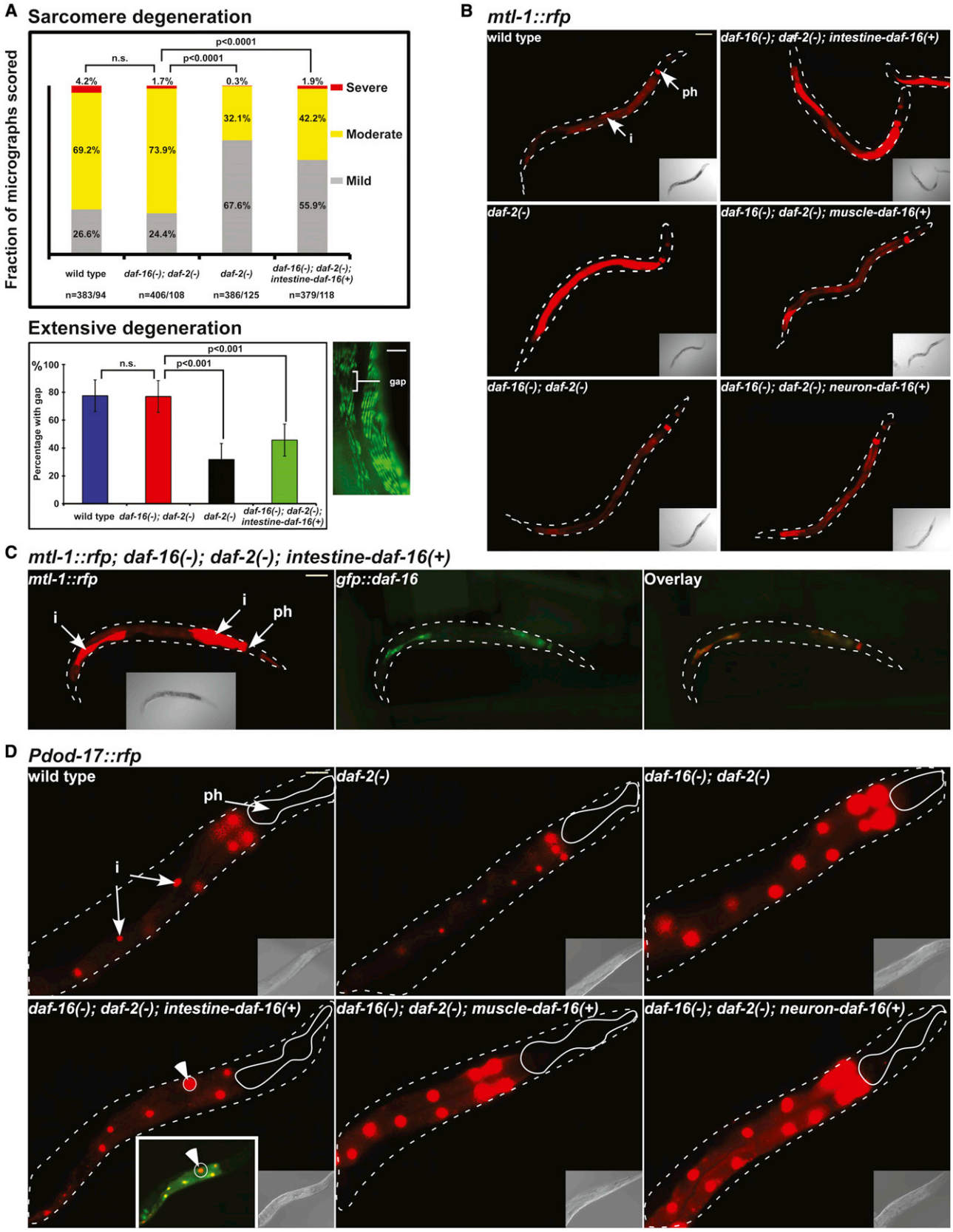
Insulin and IGF-1 signaling pathways influence the rate of aging in many species, and they appear to affect human aging, as well (Barzilai et al., 2012; Kenyon, 2010). However, the mechanisms by which insulin and IGF-1 hormones coordinate the aging of individual tissues are poorly understood. In *C. elegans*, the life-span extension produced by reduced insulin/IGF-1 signaling requires the FOXO transcription factor DAF-16 (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). DAF-16/FOXO is an important longevity regulator, as its disruption accelerates the rate of normal aging (Garigan et al., 2002; Haithcock et al., 2005)—and increasing its activity can extend life span (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). Its function in life-span regulation may be ancient: forkhead-box transcription factors can extend life span in yeast (Postnikoff et al., 2012), and FOXO proteins can extend the life span of *Drosophila* (Giannakou et al., 2004; Hwangbo et al., 2004) and possibly humans (Barzilai et al., 2012).

DAF-16 extends the life span of *C. elegans* insulin/IGF-1-pathway mutants by affecting the expression of stress-response, metabolic, innate-immunity, signaling, germline, and other genes (Curran et al., 2009; Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003; Wang et al., 2008). Presumably, these genes hold much of the answer to the question of how life span can be extended; yet, we know little about their positions in this regulatory network. How are their activities distributed among the different tissues? In which tissues are their activities altered in long-lived mutants? Is each gene regulated directly by DAF-16, or are intermediate, possibly intercellular, factors required? These are fascinating, system-wide questions that address the function of this endocrine pathway as a whole.

DAF-16 is expressed in many tissues, raising the possibility that it directly regulates many genes in a strictly cell-autonomous fashion. Consistent with this idea, expressing *daf-16(+)* exclusively in the intestine, muscles or neurons of a *daf-2(−)* mutant switches on the *sod-3* superoxide dismutase gene in that tissue alone (Libina et al., 2003). The *sod-3* promoter contains consensus DAF-16/FOXO-binding elements (DBEs) (Biggs et al., 2001; Furuyama et al., 2000; Pierrou et al., 1994), which bind DAF-16 both in vitro (Furuyama et al., 2000) and in vivo (Oh et al., 2006).

DAF-16 activity can also influence cells at a distance. First, in a process we call FOXO-to-FOXO signaling, increasing *daf-16* gene dosage in one tissue (neurons, intestine) upregulates DAF-16 activity in other tissues (Libina et al., 2003). Intestinal DAF-16 mediates FOXO-to-FOXO signaling, at least in part, by downregulating an intestinal insulin gene (Murphy et al., 2007). The *C. elegans* intestine is the animal's entire endoderm, also functioning as the adipose tissue, liver, and pancreas. Consistent with this, overexpressing *dFOXO* specifically in the fat body of *Drosophila* reduces the expression of the insulin-like peptide gene *dilp-2* in neurons and reduces insulin/IGF-1 signaling in peripheral tissues (Giannakou et al., 2004; Hwangbo et al., 2004).

DAF-16 also appears to initiate a fundamentally different type of cross-tissue communication, one that does not require DAF-16 activity in responding tissues. Expressing *daf-16* exclusively in the intestine of a *daf-16(−); daf-2(−)* double mutant


A
Sarcomere degeneration
Fraction of micrographs scored
p<0.0001
n.s.
p<0.0001
4.2%
1.7%
0.3%
1.9%
Severe
Moderate
Mild
69.2%
73.9%
32.1%
42.2%
67.6%
55.9%
26.6%
24.4%
wild type
daf-16(-); daf-2(-)
daf-2(-)
daf-16(-); daf-2(-); intestine-daf-16(+)
n=383/94
n=406/108
n=386/125
n=379/118
Extensive degeneration
p<0.001
n.s.
p<0.001
%
Percentage with gap
wild type
daf-16(-); daf-2(-)
daf-2(-)
daf-16(-); daf-2(-); intestine-daf-16(+)
gap
B
mtl-1::rfp
wild type
ph
i
daf-16(-); daf-2(-); intestine-daf-16(+)
daf-2(-)
daf-16(-); daf-2(-); muscle-daf-16(+)
daf-16(-); daf-2(-)
daf-16(-); daf-2(-); neuron-daf-16(+)
C
mtl-1::rfp; daf-16(-); daf-2(-); intestine-daf-16(+)
mtl-1::rfp
i
i
ph
gfp::daf-16
Overlay
D
Pdod-17::rfp
wild type
ph
i
daf-2(-)
daf-16(-); daf-2(-)
daf-16(-); daf-2(-); intestine-daf-16(+)
daf-16(-); daf-2(-); muscle-daf-16(+)
daf-16(-); daf-2(-); neuron-daf-16(+)

extends life span by 50%–70%, and expressing *daf-16* only in nonintestinal tissues extends life span by 50% (Libina et al., 2003). To a lesser extent, DAF-16 can also act exclusively in neurons (Libina et al., 2003) or skin (this study) to increase life span, as well. The finding that DAF-16 is not absolutely required in any one tissue to extend *C. elegans*' life span implies that DAF-16 can extend life span by modulating expression of genes encoding downstream hormones or metabolites that act independently of *daf-16* to delay aging in responding tissues. If so, then genes like *sod-3*, which require DAF-16 cell autonomously for their expression, might be the exception and not the rule.

To address these questions more systematically, in vivo, we surveyed longevity genes that are either up- or downregulated by DAF-16 in long-lived *daf-2(-)* mutants to ask (1) in which tissues they are expressed; (2) whether DAF-16 regulates them in a strictly cell-autonomous fashion, or remotely, at a distance; and (3) what mechanisms control their tissue-specific expression.

RESULTS

DAF-16 Can Affect Tissue Aging at a Distance

daf-2(−) mutants expressing *daf-16* only in the intestine are long-lived (Libina et al., 2003) (Table S3 and Figure S6A), but is this increased longevity correlated with a more youthful appearance of individual tissues? Aging *C. elegans* muscles resemble those of human sarcopenia patients, in which muscle filaments fragment and break. We found that expressing *daf-16* exclusively in the intestine of *daf-16(−); daf-2(−)* mutants reduced this muscle deterioration (Figures 1A and 6A) and improved body movement (Figure S6B). Thus, intestinal DAF-16 can act at a distance to delay the aging of *daf-16(−)* muscles.

Analysis of DAF-16-Regulated Genes In Vivo

To better understand the DAF-16 regulon at the tissue level, we studied, in vivo, a diverse collection of DAF-16-regulated genes that we had identified in gene-expression arrays comparing long-lived *daf-2(−)* mutants to *daf-16(−); daf-2(−)* mutants (Murphy et al., 2003). Our set included stress-resistance, chaperone, signaling, innate immunity, and metabolic genes whose RNAi knockdown influenced life span, as well as additional genes for which RNAi analysis had not been performed.

We analyzed the expression of each gene by using ~1–3 kb upstream DNA to drive expression of red fluorescent protein (RFP), or, in a few cases, green fluorescent protein (GFP). We first examined each reporter for its response to *daf-2* RNAi, which stimulates DAF-16/FOXO's transcriptional activity. Twenty of the forty-four new genes we tested exhibited the predicted (up or down) response to *daf-2* inhibition (Table S1). The 24 negatives could include microarray false positives, or genes influenced by regulatory elements downstream of the promoter. Consistent with this, the *mtl-1* transcriptional fusion exhibited little or no response to *daf-2* RNAi, but a translational fusion driven by the same promoter responded very strongly (Figures 1B and S1C).

Genes Upregulated in Long-Lived *daf-2(−)* Mutants

Fifteen of the twenty *daf-2*-sensitive reporters were upregulated under *daf-2(−)* conditions (Table S1). One, *lys-7*, encodes an innate-immunity lysozyme. Several, like *sod-3,* were stress-resistance genes: *mtl-1* encodes a metallothionein protein that confers resistance to heavy metals. *hsp-12.6* and *hsp-16.2* encode small heat-shock proteins that contribute to the isotonic stress resistance of insulin/IGF-1-pathway mutants (Lamitina and Strange, 2005), as do four additional proteins, encoded by *hgo-1* (homogentisate 1,2-dioxygenase), *tps-1* and *tps-2* (trehalose-6-phosphate synthases), and *tre-4* (trehalase) (Lamitina and Strange, 2005). In addition, several genes encode metabolic enzymes, including *gpd-2* (glyceraldehyde-3-phosphate dehydrogenase), *nnt-1* (nicotinamide nucleotide transhydrogenase), *dod-11* (sorbitol dehydrogenase), and *dod-8* (17 beta-hydroxysteroid dehydrogenase). *F09F7.7* and *ZK384.3* encode proteins that are similar to human α-ketoglutarate-dependent dioxygenase and gastricsin, respectively. Finally, *sma-10* encodes a TGF-β-pathway member.

daf-2 and *daf-16* act exclusively during adulthood to influence aging (Dillin et al., 2002), so we examined expression during early adulthood. In the wild-type, 13 of the 15 genes were expressed in more than one tissue, and, with few exceptions (*dod-8*, *tps-2*, and *nnt-1*), their expression increased in each of those tissues under *daf-2(−)* conditions (Table S1). Interestingly, all but one of these upregulated genes was expressed and upregulated in the intestine. Moreover, three genes whose functions contribute to the longevity of *daf-2(−)* mutants (*lys-7*, *mtl-1*, and *hsp-16.2*)

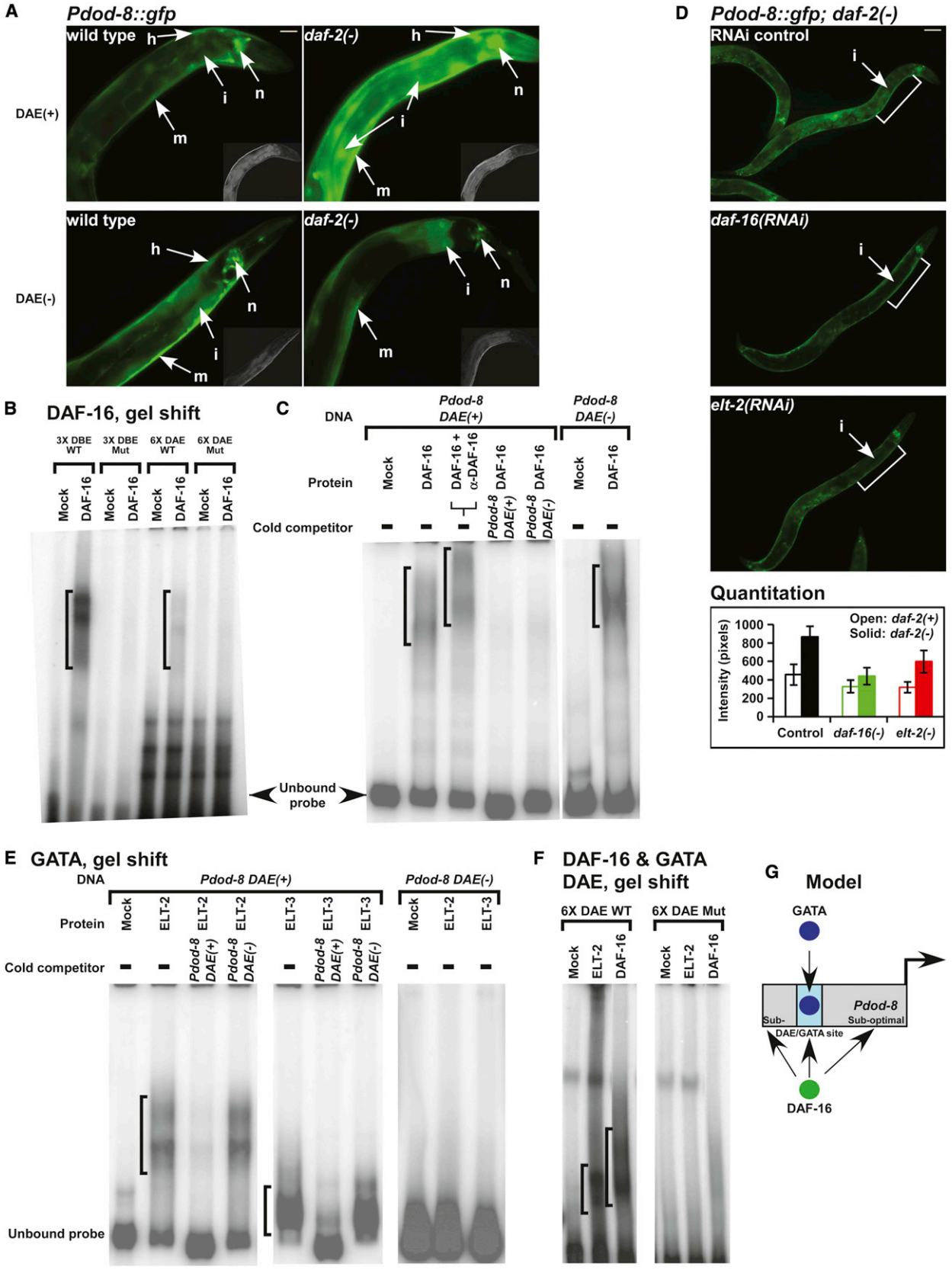
Figure 1. DAF-16 Acts Cell Nonautonomously to Regulate Muscle Aging and Autonomously to Regulate *mtl-1* and *dod-17* Expression

(A) Intestinal DAF-16 protects *daf-16(−)* muscles from age-dependent deterioration. Top: Extent of sarcomere degeneration on day 10 of adulthood (micrographs scored/animals analyzed) (see Supplemental Experimental Procedures) (Mann-Whitney-Wilcoxon test of all data points; n.s., not significant). Bottom: Percentages of animals with extensive degeneration (Class C, as defined by Herndon et al. [2002]) (Student's t test of independent experiments). A gap in the sarcomere of a day 10 Class C animal is shown; 250X magnification. Scale bar: 50 μm. Note that intestinal *daf-16(+)* does not fully restore muscle quality to that seen in nontransgenic *daf-16(+); daf-2(−)* mutants, just as it does not extend life span to the extent seen in *daf-16(+); daf-2(−)* animals.

(B) Left panels: A *mtl-1::rfp* translational reporter is expressed mainly in the pharynx (ph) and intestine (i) of wild-type, and is upregulated in the intestines of *daf-2(−)* mutants in a *daf-16*-dependent manner. Right panels: intestinal GFP-tagged DAF-16 upregulates *mtl-1::rfp* only in the intestine. Muscle or neuronal *daf-16* does not affect *mtl-1::rfp* expression. The body is outlined. *daf-2(e1370)* and *daf-16(mu86)* mutations were used. Young adults, 100X magnification. Scale bar: 130 μm.

(C) The *ges-1* promoter, which drives *gfp::daf-16* expression, is expressed in a mosaic fashion in the intestine (middle panel). DAF-16 turns on *mtl-1::rfp* expression in the same cells (left panel). Young adults, 100X magnification. Scale bar: 130 μm.

(D) Top panels: A nuclear-localized *Pdod-17::rfp* transcriptional reporter is expressed mainly in the intestine (i) of wild-type and is downregulated in the intestine of *daf-2(−)* mutants and upregulated in *daf-16(−); daf-2(−)* mutants. Bottom panels: expression of *daf-16* in the intestine, but not muscles or neurons, suppresses *Pdod-17::rfp* expression. Inset: *daf-16* expression (green) is inversely correlated with *dod-17* expression (red). Note that *Pdod-17::rfp* expression is higher in an intestinal cell that does not express *gfp::daf-16* (arrowhead). The body and pharynx (ph) are outlined. Young adults, 250X magnification. Scale bar: 50 μm. Representative images from 2 or more experiments are shown for all figures.


A
Pdod-8::gfp
wild type
daf-2(-)
DAE(+)
DAE(-)
h
i
n
m
B
DAF-16, gel shift
3X DBE WT
3X DBE Mut
6X DAE WT
6X DAE Mut
Mock
DAF-16
C
DNA
Pdod-8 DAE(+)
Pdod-8 DAE(-)
Protein
Cold competitor
DAF-16 + α-DAF-16
Unbound probe
D
Pdod-8::gfp; daf-2(-)
RNAi control
daf-16(RNAi)
elt-2(RNAi)
Quantitation
Intensity (pixels)
Open: daf-2(+)
Solid: daf-2(-)
Control
daf-16(-)
elt-2(-)
E
GATA, gel shift
ELT-2
ELT-3
F
DAF-16 & GATA DAE, gel shift
6X DAE WT
6X DAE Mut
G
Model
GATA
Pdod-8
Sub-
Sub-optimal
DAE/GATA site
DAF-16

(Murphy et al., 2003; Walker and Lithgow, 2003) were expressed and upregulated only in the intestine.

Genes Downregulated in Long-lived *daf-2(–)* Mutants

DAF-16 also downregulates genes in *daf-2(–)* mutants, and RNAi knockdown of certain downregulated genes lengthens wild-type life span (Murphy et al., 2003). We analyzed: *pept-1*, which encodes a predicted dipeptide transporter; *vit-5*, which encodes a putative lipid transporter related to vertebrate vitellogenins and mammalian ApoB-100, a core LDL particle constituent; *his-24*, which encodes a *C. elegans* H1 linker histone; *ZC416.6*, similar to human leukotriene A-4 hydrolase; and one gene with unknown function, *dod-17*. Remarkably, all five genes were expressed only in the intestine (or intestine plus pharynx) (Table S1).

DAF-16 Regulates Many Genes Cell Autonomously

Next, we chose nine genes with strong *daf-2(–)* induction ratios for DAF-16 cell-autonomy studies. We introduced transgenic reporters for these genes into *daf-16(–); daf-2(–)* mutants in which *daf-16* was expressed in only one tissue: intestine, neurons, or muscles. We confirmed the tissue specificity of *daf-16* expression by using a functional GFP::DAF-16 protein fusion (Libina et al., 2003). Surprisingly, as with *sod-3* (Libina et al., 2003); used as a control), six of these nine genes were regulated in a strictly cell-autonomous fashion (*lys-7*, *mtl-1*, *hgo-1*, *gpd-2*, *nnt-1*, and *dod-17*). The demonstration of cell autonomy was particularly striking in the intestine, because the *ges-1* intestinal promoter we used did not fire evenly in all cells. We observed a close correlation between GFP::DAF-16 and reporter expression among individual intestinal cells with each of these six genes (Figure 1C, *mtl-1*; Figure 1D, *dod-17*; Figure S1B, *lys-7*), plus *sod-3* (Figure S1A).

DAF-16 Binds Multiple DNA Sequences to Upregulate Gene Expression

Does DAF-16 bind directly to genes that it regulates cell autonomously? All but one of the 20 new DAF-16-regulated transgenes we analyzed contain at least one copy of the canonical DAF-16-binding element, consistent with this possibility. Previously, Schuster et al. (2010) demonstrated DAF-16 binding to the DBE-containing genes *hsp-12.6*, *hgo-1*, *tps-1*, *gpd-2*, and *F09F7.7* in chromatin profiling experiments. We tested for DAF-16's binding to our eleven most highly regulated genes, plus *sod-3*, in chromatin immunoprecipitation (ChIP) experiments. In our experiments, DAF-16 bound to *sod-3*, *dod-8*, *hsp-12.6*, and *tps-1* preferentially in *daf-2(–)* mutants in both of two experiments, and to *mtl-1*, *nnt-1*, *hgo-1*, *dod-11*, and *dod-17* in one of two experiments (see Figure S4D for details). We also examined the online modENCODE database for DAF-16 binding profiles for these same genes (http://intermine.modencode.org/release-30/report.do?id=64000352). Even in wild-type, in which DAF-16 is only partially activated, DAF-16 bound to genomic regions of ten of these twelve genes (*sod-3*, *dod-8*, *mtl-1*, *gpd-2*, *nnt-1*, *hgo-1*, *tps-1*, *tps-2*, *dod-11*, and *hsp-12.6*) (Figure S4D).

The one *daf-2*/*daf-16*-responsive reporter that lacked a DBE contained a small, 0.5 kb, *dod-8* promoter sequence (Figures 2A and S2). This reporter was noteworthy, as, to our knowledge, DAF-16 has not been shown to bind any non-DBE sites in *C. elegans.* One potential DAF-16-binding site in this fragment was the so-called DAE (DAF-16-associated element, CTTATCA), as this sequence is overrepresented in promoter regions of DAF-16-regulated genes (Murphy et al., 2003). The DAE was significant in vivo, as deleting it prevented a *daf-2* mutation from upregulating *Pdod-8::gfp* expression (Figure 2A). In gel-shift assays, DAF-16 bound to an oligonucleotide that contained six tandem wild-type copies (but not six mutant copies) of this site (Figure 2B). Thus, DAF-16 can bind the DAE.

Unexpectedly, we found that DAF-16 was able to bind to the 0.5 kb *dod-8* promoter fragment lacking the DAE (but not to control plasmid DNA) (Figures 2C and S3B). Thus, we looked for additional DAF-16-binding sites. Certain suboptimal DBE-like sites with a conserved core sequence "AAACAA" have been observed in upstream sequences of several FOXO-regulated genes (Santo et al., 2006; Tran et al., 2002). Some of these are functionally significant (Tran et al., 2002). The *dod-8* promoter contains different suboptimal DBEs (Figure S3A). We found that DAF-16 bound to *dod-8* promoter-derived oligonucleotides that contained these motifs, but not to random oligonucleotides (Figure S3A). Removing these sites in the transgene prevented hypodermal *Pdod-8::gfp* expression

Figure 2. DAF-16 and GATA Factors Bind to *dod-8* Promoter Sequences In Vitro and Regulate *dod-8* In Vivo

(A) The 504 bp *dod-8* promoter fragment drives *gfp* (cytoplasmic) expression in the intestine (i), hypodermis (h), body-wall muscles (m), and neurons (n) of wild-type and is upregulated in most tissues of *daf-2(–)* mutants. Deletion of the DAE did not have marked effects on *Pdod-8::gfp* expression in wild-type but significantly attenuated intestinal expression and abolished hypodermal expression in *daf-2(–)* mutants (right, bottom panel). Young adults, 250X magnification. Scale bar: 50 μm.

(B) Bacterially expressed GST-tagged DAF-16 gel-shifted an oligonucleotide containing three DBEs or, to a lesser extent, six DAEs. DBE and DAE point mutations abolished the binding. Shifted oligos are highlighted. Mock: purified GST. Representative autoradiograph from two or more experiments is shown for all figures.

(C) DAF-16 gel-shifted the 504 bp *dod-8* promoter fragment independently of the DAE. Left block: DAF-16 binding, which could be super-shifted with a DAF-16 antibody, could be inhibited with cold competitor, a *dod-8* promoter fragment that lacked the DAE. Right block: DAF-16 still bound to the *dod-8* promoter fragment following DAE deletion.

(D) Knockdown of *daf-16* or *elt-2*/GATA significantly attenuated *Pdod-8::gfp* induction in the intestine of *daf-2(–)* mutants. Young adults were photographed (100X) using a low exposure to avoid signal saturation. Scale bar: 130 μm. Lower panel: The GFP signal in the anterior quarter of the intestine ("i," as indicated) was quantified. Bars, mean value ± SD. *daf-2(+)* background: RNAi control, n = 36 (animals); *daf-16(RNAi)*, n = 36, p = 2.19E−07 (Student's t test, versus control); *elt-2(RNAi)*, n = 36, p = 2.32E-08. *daf-2(–)* background: RNAi control, n = 24; *daf-16(RNAi)*, n = 20, p = 2.69E−17; *elt-2(RNAi)*, n = 24, p = 3.21E−10.

(E) Both ELT-2 and ELT-3 gel-shifted DAE/GATA site-containing DNA. Binding could be competed away by a wild-type *dod-8* promoter fragment, but not a mutant DAE/GATA(–)-promoter fragment. Likewise, neither ELT-2 nor ELT-3 could gel-shift the *dod-8* promoter fragment lacking the DAE/GATA site. Mock: copurified proteins produced by the empty vector pET-28.

(F) Both ELT-2 and DAF-16 gel-shifted wild-type but not mutant DAE/GATA sequences. Mock: purified GST.

(G) Model for coregulation of *dod-8* by DAF-16 and GATA factors. Note that DAF-16 can bind additional, unidentified promoter sites, as well.

Table 1. ELT-2 and ELT-3 Are Required for Expression of Some DAF-16-Regulated Genes

Gene Reporter	Expression under *daf-2(−)* Conditions	Promoter Used for Analysis				Expression upon RNAi		
		Length (kb)	DBE	DAE	GATA	*daf-16(−)*	*elt-2(-)*	*elt-3(-)*
sod-3	Up, intestine, hypodermis, muscles, neurons	1.1	4 + 1 sub	0	4	Down	Down (intestine, modest)	No Change
dod-8(*)	Up, intestine, hypodermis, muscles, neurons	0.5	0*	1	1	Down	Down (intestine)	Down (hypodermis)
lys-7	Up, intestine	3.1	7 + 4 sub	1	10	Down	Down (intestine)	No Change
mtl-1	Up, intestine	3.6	5 + 15 sub	2	12	Down	Down (intestine)	No Change
gpd-2	Up, intestine	1.1	1	0	2	Down	Down (intestine, modest)	No Change
nnt-1	Up, intestine, hypodermis	1.9	10 + 6 sub	0	8	Down	Down (intestine)	Down (hypodermis)
hgo-1	Up, intestine, hypodermis	2.9	4 + 9 sub	1	12	Down	Down (intestine)	Down (hypodermis)
tps-1	Up, intestine, muscles	2.8	6 + 2 sub	1	10	Down	No Change	No Change
tps-2	Up, hypodermis, muscles	2.9	5 + 8 sub	1	12	Down	No Change	Down (hypodermis)
dod-11	Up, intestine, hypodermis, muscles	5.3	6 + 14 sub	2	10	Down	Down (intestine, modest)	No Change
hsp-12.6	Up, intestine, hypodermis, muscles	2.7	9 + 13 sub	0	11	Down	Down (intestine)	No Change
dod-17	Down, intestine	2.6	4 + 7 sub	0	10	Up	No Change	No Change

The table lists the 12 genes analyzed for ELT-2 and ELT-3 influence. These genes were chosen because their expression was most strongly changed by *daf-2* RNAi. DBE, RTAAAYA, R = A/G, Y = C/T; DAE, CTTATCA; GATA, WGATAR, W = A/T; sub, suboptimal DBE (e.g., TAAAACAA and TTGTTTGT [Santo et al., 2006; Tran et al., 2002]). RNAi experiments were initiated at the L4 stage, 25°C, and RNAi-treated animals were analyzed as young adults. At least two independent RNAi experiments were performed, and at least ten transgenic animals were analyzed for each reporter. *: There are two DBEs ~0.66 kb upstream of the *dod-8* translational start site, not included in the 0.5 kb promoter fragment we analyzed.

in vivo and attenuated its induction in the intestine and muscles of *daf-2(−)* mutants (Figure S3C). Thus, these sites appear to play an important role in vivo.

Unexpectedly, DAF-16 still bound to the *dod-8* promoter in the absence of the DAE site and all three noncanonical binding sites (Figure S3B). Thus, additional site(s) may contribute to *daf-16*-dependent *dod-8* expression in vivo, especially since, compared with the canonical DBE, DAF-16 appeared to have much lower affinity for the DAE and other noncanonical sites (Figures 2B and S3A).

GATA Factors Bind to the DAE and Regulate the Expression of *Pdod-8::gfp*

Since DAF-16 bound many sites in the *dod-8* promoter fragment, we wondered why the DAE was so important in vivo. The DAE is the reverse complement of the mammalian GATA factor binding site (Plumb et al., 1989). Thus, the DAE site in *dod-8* might also be recognized by GATA factors. *C. elegans* has at least fourteen GATA-factor genes (Kormish et al., 2010; Maduro and Rothman, 2002). Using RNAi, we knocked down ten characterized GATA factors (*elt-1*, *elt-2*, *elt-3*, *elt-5(egl-18)*, *elt-6*, *elt-7*, *end-1*, *end-3*, and *med-1/med-2*). Knocking down *elt-2*, which is expressed in the intestine, specifically decreased intestinal expression of *Pdod-8::gfp* (Figure 2D). In contrast, knocking down *elt-3,* which is expressed in the hypodermis but not the intestine (Gilleard et al., 1999; Tonsaker et al., 2012) (data not shown), specifically decreased hypodermal expression (Figure S4C and Table 1).

Both ELT-2 and ELT-3 bound to the wild-type *dod-8* promoter in vitro, but not to a DAE-mutant *dod-8* promoter (Figure 2E) or DAE-mutant oligonucleotide (Figure 2F). As expected, the *dod-8* promoter could bind GATA factors and DAF-16 at the same time (Figures S4A and 2G). In vivo, knocking down either *daf-16* or *elt-2* did not further reduce expression of the *Pdod-8::gfp* reporter that lacked the DAE (Figure S4B). Together, these results suggested that ELT-2 and ELT-3 recognize the DAE/GATA site in vivo, thereby promoting expression of *dod-8* in *daf-2(−)* mutants.

GATA Factors and DAF-16 Coregulate a Subset of DAF-16 Target Genes

We tested the 12 reporters that exhibited the greatest change under *daf-2(−)* conditions for *elt-2* and *elt-3* dependency. Knockdown of the intestinal GATA-factor gene *elt-2* affected 9 of these 12 reporters (*sod-3*, *dod-8*, *lys-7*, *mtl-1*, *gpd-2*, *nnt-1*, *hgo-1*, *dod-11*, and *hsp-12.6*), and only in the intestine (Table 1). Likewise, knockdown of the hypodermal factor *elt-3* affected *dod-8*, *nnt-1*, *hgo-1*, and *tps-2* reporters, and only in the hypodermis. Using qPCR, we found that RNAi inhibition of *elt-2*, but not *elt-3*, resulted in significant attenuation of a subset of intestine-expressed DAF-16 target genes in *daf-2(−)* mutants, including *sod-3*, *lys-7*, *mtl-1*, *nnt-1*, and *hgo-1* (Figure 3A). Notably, all of the gene reporters that responded to GATA-factor knockdowns contained at least one DAE/GATA site in their promoters. Together, these results suggested that DAF-16 and tissue-specific GATA factors collaborate to establish tissue-specific expression of multiple downstream target genes.

The strong influence that the intestinal ELT-2 GATA factor had on the expression of DAF-16 targets suggested that *elt-2* might be required for the long life spans of *daf-2(−)* mutants. *elt-2* is essential for development of the intestine (Fukushige et al., 1998; Kormish et al., 2010). We found that adult-only RNAi

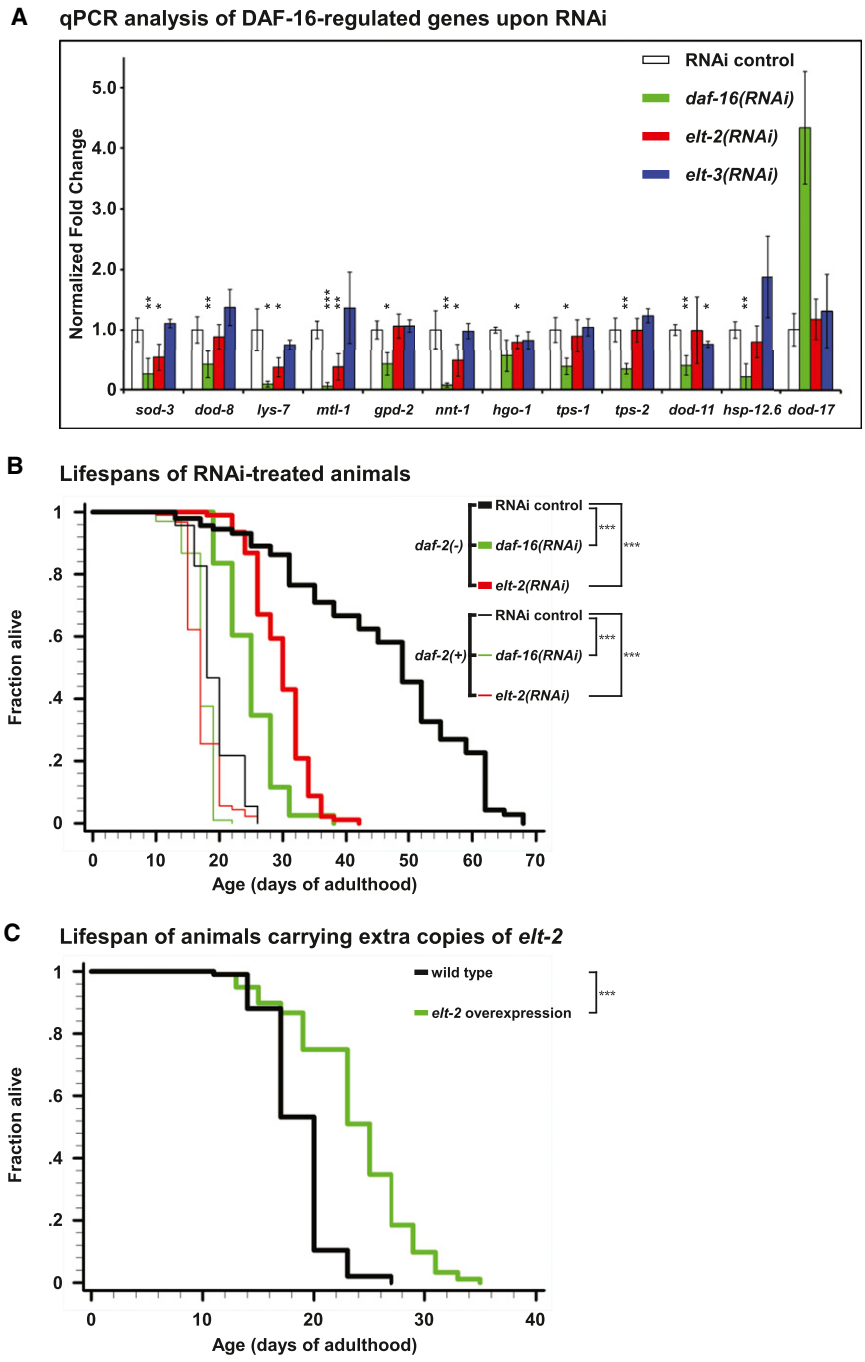


Figure 3. The ELT-2 GATA Factor Regulates DAF-16 Target Genes and Extends Life Span

(A) *elt-2* knockdown affected the expression of a subset of DAF-16-regulated genes in *daf-2(–)* mutants. RNAi-sensitive *rrf-3(–)* mutants were used for RT-qPCR analysis. Bars, mean value ± SD, four biological replicates, technical triplicates. Gene expression was normalized to the gene *nhr-23* (Supplemental Experimental Procedures). Student's t test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(B) Knockdown of *daf-16* or *elt-2* shortens the life span of *daf-2(–)* mutants. Representative data from at least six independent RNAi experiments are shown (see Table S2). Log-rank test, ***$p < 0.001$.

(C) Increasing *elt-2* gene dosage increases life span. Life span was increased (7%–30%) in two independent lines (see Table S2). Log-rank test, ***$p < 0.001$.

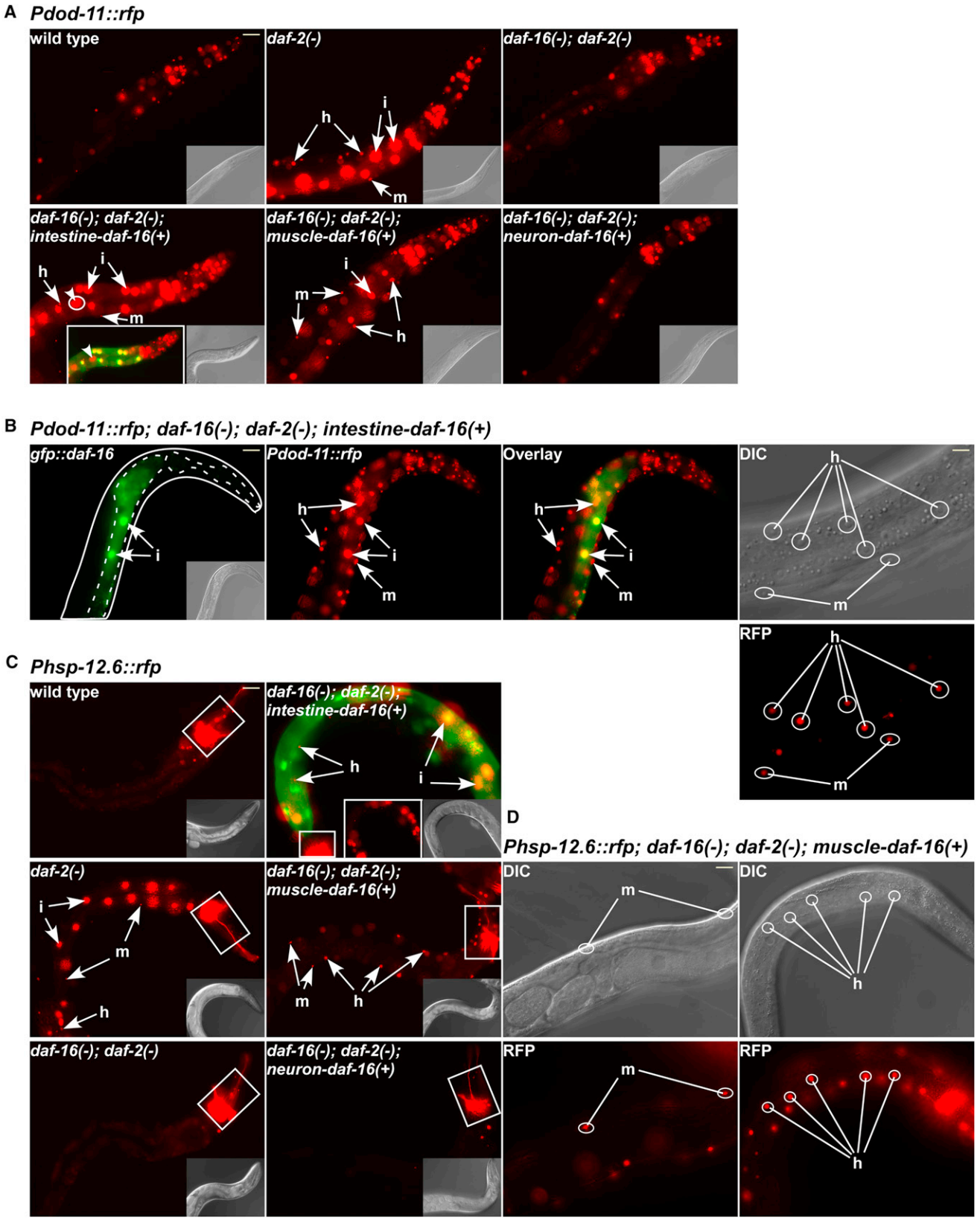
inhibition of *elt-2,* but not other GATA factors (Table S2), shortened the life span of wild-type by ~10%–20%, but consistently produced a stronger, 30%–45% shortening of life span, and reduced heat-stress resistance in *daf-2(–)* mutants (Figure 3B and Table S2). *elt-2* inhibition also shortened the life span of calorically restricted *eat-2* mutants and germline-less *glp-1* mutants substantially (by 40%–50% and by 25%, respectively), but it did not preferentially shorten the long life spans of respiration mutants (Table S2). Finally, increasing *elt-2* gene dosage (Fukushige et al., 1999) increased the life span of wild-type by 7%–28% (Figure 3C and Table S2). Thus, activity of the intestinal GATA-factor ELT-2 during adulthood has an important influence on the life span of *C. elegans*.

elt-3 played an important role in the upregulation of four DAF-16-controlled genes in the hypodermis (skin). Consistent with this, we found that hypodermal-only *daf-16* expression was able to increase the life span of a *daf-16(–); daf-2(–)* mutant by 16% and 32% in two experiments (Figure S7). However, when we removed *elt-3* in *daf-2(–)* mutants, we did not observe a decrease in life span (Figures S5A–S5C). This apparent paradox suggests that DAF-16 can regulate important hypodermal life-span genes independently of ELT-3.

DAF-16 Regulates Some Target Genes Cell Nonautonomously

Three of the nine genes we analyzed for cell autonomy, the sorbitol dehydrogenase gene *dod-11*, the small heat-shock protein gene *hsp-12.6*, and the steroid dehydrogenase gene *dod-8*, were regulated cell nonautonomously by DAF-16.

***dod-11*:** In *daf-2(–)* mutants expressing *daf-16(+)* only in the intestine, expression of *Pdod-11::rfp* was induced in the hypodermis and muscles in multiple independent lines (Figures 4A and 4B). (Again, we confirmed the tissue specificity of GFP::DAF-16 using fluorescence microscopy.) Thus, DAF-16 causes intestinal cells to make a signal that can activate *dod-11* independently of *daf-16* in other tissues. Within the intestine itself, GFP::DAF-16-positive cells generally expressed *Pdod-11::rfp*, but we observed exceptions (Figure 4A). Thus, DAF-16 might regulate *dod-11* in a partially cell-nonautonomous fashion within the intestine, as well. The intestine was not the only tissue capable of affecting *dod-11* expression elsewhere in the animal: animals expressing


A
Pdod-11::rfp
wild type
daf-2(-)
daf-16(-); daf-2(-)
daf-16(-); daf-2(-);
intestine-daf-16(+)
daf-16(-); daf-2(-);
muscle-daf-16(+)
daf-16(-); daf-2(-);
neuron-daf-16(+)
B
Pdod-11::rfp; daf-16(-); daf-2(-); intestine-daf-16(+)
gfp::daf-16
Pdod-11::rfp
Overlay
DIC
RFP
C
Phsp-12.6::rfp
wild type
daf-16(-); daf-2(-);
intestine-daf-16(+)
daf-2(-)
daf-16(-); daf-2(-);
muscle-daf-16(+)
daf-16(-); daf-2(-)
daf-16(-); daf-2(-);
neuron-daf-16(+)
D
Phsp-12.6::rfp; daf-16(-); daf-2(-); muscle-daf-16(+)
DIC
DIC
RFP
RFP
h
i
m

daf-16 only in muscles exhibited *dod-11* induction in the muscles, intestine, and hypodermis (Figure 4A).

***hsp-12.6*:** Intestine-expressed *daf-16* induced *hsp-12.6* expression in the intestine as well as in *daf-16(–)* tissues, such as the hypodermis (Figure 4C). In addition, muscle-expressed *daf-16* was able to induce the *hsp-12.6* reporter in both muscles and hypodermis (Figures 4C and 4D).

***dod-8*:** In two independent transgenic lines, intestinal DAF-16 activity strongly attenuated *dod-8* reporter expression in the hypodermis and muscles (Figure S2). Together, these findings indicate that DAF-16 action in any of several tissues can influence gene expression independently of *daf-16*, either positively or negatively, elsewhere in the animal. These findings provide molecular correlates for DAF-16's ability to affect the aging of tissues in which it is not expressed.

The Lipid-Gene Regulator MDT-15 and Longevity

How does DAF-16 influence gene expression at a distance? To address this question, we used RNAi to test ~250 DAF-16-regulated genes (twice, in two independent experiments) for their effects on *dod-11* expression in *daf-2(–)* mutants, and in *daf-2(–)* mutants expressing *daf-16(+)* only in the intestine. One RNAi clone, for *mdt-15*, sharply decreased *dod-11* expression in both strains (Figures 5A and 5B). *mdt-15* transcriptional reporters (Taubert et al., 2006) (obtained from the Genome BC *C. elegans* Gene Expression Consortium), as well as RNA in situ hybridizations (The Nematode Expression Pattern Database), displayed *mdt-15* expression in the intestine and some head neurons but not in the muscles or hypodermis. We observed a similar tissue distribution of *mdt-15* reporter expression in *daf-2(RNAi)* strains. Thus, *mdt-15* may help to mediate DAF-16's action at a distance.

MDT-15 is a transcriptional mediator that regulates expression of lipid and other metabolic genes (Taubert et al., 2006, 2008). *mdt-15* is upregulated by DAF-16 in *daf-2(–)* mutants (Murphy et al., 2003) and in long-lived germline-defective animals (McCormick et al., 2012). *mdt-15* RNAi shortened the life span of wild-type by ~20% (Figure 5C and Table S4), as reported (Taubert et al., 2006). However, we found that *mdt-15* RNAi shortened the life span of *daf-2(–)* mutants by ~45%. Similarly, *mdt-15* RNAi shortened the life span of *daf-16(–); daf-2(–)* mutants by ~10%–20%, but it shortened the life span of *daf-16(–); daf-2(–)* mutants expressing *daf-16* in the intestine by ~30% (Figures 5D and Table S4). Thus, *mdt-15* is important for wild-type longevity, and even more important for the extended life spans of *daf-2(–)* mutants. At the tissue level, *mdt-15* RNAi reduced the ability of intestinal *daf-16(+)* to delay muscle deterioration (Figure 6A), while having no significant effects on the sarcomeres of *daf-16(–); daf-2(–)* mutants. However, *mdt-15* may affect additional processes required for movement, as *mdt-15* RNAi decreased the motility of both *daf-16(–)* and *daf-16(+)* animals (Figure S6C).

DAF-16 Can Act at a Distance to Protect Animals from Amyloid Paralysis

When expressed in *C. elegans*' muscles, the human Alzheimer's protein Aβ(1-42) aggregates and paralyzes the animals during early adulthood (Link, 1995). This paralysis is attenuated by insulin/IGF-1-pathway mutations (Cohen et al., 2006; Florez-McClure et al., 2007). We wondered whether DAF-16 could also act at a distance to counteract Aβ toxicity. In multiple independent lines, we found that Aβ-containing *daf-16(–); daf-2(–)* adults expressing intestinal *daf-16(+)* moved much better than did Aβ-containing wild-type animals or *daf-16(–); daf-2(–)* mutants (Figures 6B, 6C, and S6E and Table S3). (As a control, we introduced the Aβ transgene back into wild-type and found that it still induced paralysis [Table S3].) The ability of DAF-16 to counteract Aβ toxicity correlated with its expression levels in the intestine (Figure S6D & S6E). Thus, intestinally-expressed DAF-16 can counteract Aβ-dependent muscle dysfunction. Likewise, *mdt-15* RNAi, similar to *daf-16* RNAi, also abolished the ability of intestinal DAF-16 to delay Aβ-dependent paralysis (Figure 6D and Table S3).

DISCUSSION

Understanding how the insulin/IGF-1 endocrine system coordinates the rate of aging among different tissues is fundamentally important, as this pathway appears to influence the rate of aging throughout the animal kingdom, from worms to man. In this study, we analyzed the expression of a diverse collection of DAF-16-regulated genes in vivo to better understand how components of this signaling network map across the tissues of the animal. One could imagine two extreme cases: in one, DAF-16 would act at the end of an insulin/IGF-1 signal-transduction pathway, regulating downstream genes that affect only the health and longevity of the cells in which they are expressed. At the other extreme, since DAF-16 activity within a single tissue

Figure 4. DAF-16 Regulates *dod-11* and *hsp-12.6* Cell Nonautonomously

(A and B) (*dod-11*) In (A), top panels: *Pdod-11::rfp* is expressed in most tissues of wild-type and is upregulated mainly in the intestine (i), hypodermis (h,) and muscles (m) of *daf-2(–)* mutants in a *daf-16*-dependent manner. Bottom panel (left): intestinal DAF-16 upregulates *Pdod-11::rfp* in the intestine as well as in hypodermis and muscles (compare with *daf-16[–]; daf-2[–]*). Inset: overlay of intestinal GFP::DAF-16 (green) and *Pdod-11::rfp* (red). Note that one intestinal cell (arrowhead) does not express *gfp::daf-16* but does express *Pdod-11::rfp*. Bottom panel (middle): muscle DAF-16 upregulates *Pdod-11::rfp* in muscles as well as in the intestine and hypodermis. Young adults, 250X magnification. Scale bar: 50 μm. In (B), left three panels: Intestinal cells expressing *gfp::daf-16* and the resulting *Pdod-11::rfp* expression in intestinal (i), hypodermal (h), and muscle (m) cells are shown. Young adults, 400X magnification. Scale bar: 32 μm. Right two panels: Higher magnification of the same animal, viewed using differential interference contrast (DIC, top) or fluorescence microscopy (RFP, bottom). *Pdod-11::rfp* is expressed in the hypodermis (h) and muscles (m) when DAF-16 is activated in the intestine of *daf-2(–)* mutants. 1000X magnification. Scale bar: 13 μm. (C and D) (*hsp-12.6*) In (C), left column: *Phsp-12.6::rfp* is expressed at low levels in wild-type and is upregulated in the same tissues ("i," intestine; "h," hypodermis; "m," muscles) of *daf-2(–)* mutants in a *daf-16*-dependent manner. Right column: intestinal DAF-16 upregulates *Phsp-12.6::rfp* in the intestine as well as hypodermis (top). Note that not all *daf-16*-expressing cells (green) express *Phsp-12.6::rfp* (red) (inset: RFP only). Muscle DAF-16 upregulates *Phsp-12.6::rfp* in muscles as well as hypodermis (middle). Neuronal DAF-16 does not affect *hsp-12.6* expression (bottom). Rectangle: coinjection marker *Podr-1::rfp* expression in head neurons. Young adults, 250X magnification. Scale bar: 50 μm. In (D), DAF-16 activity in muscles upregulates *Phsp-12.6::rfp* in muscle cells (m, oblong) (left column) as well as hypodermal cells (h) (right column) is shown. Young adults, 400X magnification. Scale bar: 32 μm.

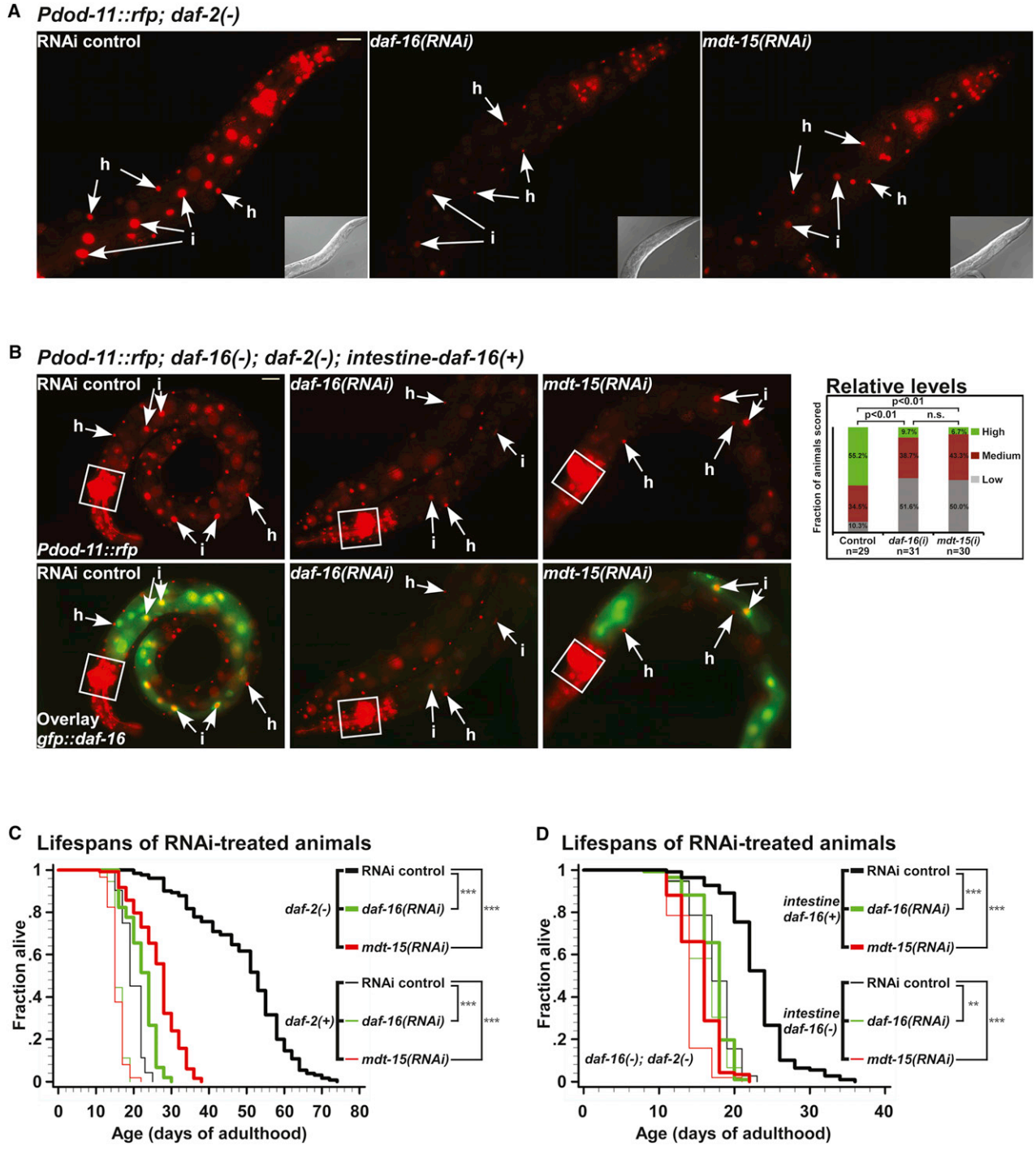


Figure 5. MDT-15 Is Required for *dod-11* Expression and for Longevity

(A and B) RNAi of either *daf-16* or *mdt-15* attenuated *Pdod-11::rfp* expression in the intestine (i) and hypodermis (h) of (A) *daf-2(–)* mutants and (B) *daf-16(–); daf-2(–)* mutants expressing *daf-16* in the intestine. *Intestine-daf-16(+): muIs199*. In (B), shown are *Pdod-11::rfp* expression (red, top panels) overlaid with intestinal GFP::DAF-16 (green, bottom panels). Relative levels of *Pdod-11::rfp* expression in RNAi-treated animals are shown on the right (Kolmogorov-Smirnov test; n.s., not significant). Note that *mdt-15* RNAi significantly reduced both the number and brightness of *Pdod-11::rfp* foci (this animal in [B] represents the "low-expression" category), despite the high level of GFP::DAF-16. Rectangle: coinjection marker *Podr-1::rfp* expression in head neurons. Young adults, 250X magnification. Scale bar: 50 μm.

(C) Knockdown of *daf-16* or *mdt-15* shortened the life span of *daf-2(–)* mutants to a greater extent than it affected wild-type (see Table S4). Log-rank test, ***$p < 0.001$.

(D) *mdt-15* RNAi shortened the life span of *daf-16(–); daf-2(–)* mutants, but had a greater life-shortening effect on *daf-16(–); daf-2(–)* animals expressing *daf-16* in the intestine (see Table S4). Log-rank test, **$p < 0.01$; ***$p < 0.001$.

can delay the aging of other tissues and increase the life span of the whole animal, DAF-16 could regulate only downstream signaling genes whose products then activate *daf-16*-independent life-extension pathways in other cells. We find that both mechanisms operate.

Limitations of Our Gene Set

Microarray analysis (Halaschek-Wiener et al., 2005; Lee et al., 2009; McElwee et al., 2003; Murphy et al., 2003) and direct DNA binding assays (Oh et al., 2006; Schuster et al., 2010) can identify *C. elegans* genes that are likely to be regulated directly versus indirectly by a transcription factor. Our in vivo imaging analysis of DAF-16-regulated genes complements these approaches and allows us to investigate the tissue specificity and cell autonomy of gene expression. The genes we analyzed have diverse functions, affecting protein homeostasis, innate immunity, and metabolism, and many have been shown to contribute to the long life spans of *daf-2(−)* mutants.

However, our analysis was biased against certain types of genes. First, neural genes: Neurons are relatively resistant to RNAi. RNAi was used in part of the microarray analysis from which we selected genes to study, and in our initial assessment of the *daf-2* dependence of transgene expression. However, previously, we showed that nonneuronal *daf-2* RNAi doubles life span, and that nonneuronal *daf-16* RNAi completely suppresses the life-span extension of *daf-2(−)* mutants (Libina et al., 2003). Moreover, expressing *daf-16* exclusively in neurons in a *daf-16(−); daf-2(−)* background produces only a small increase in life span (Libina et al., 2003). Therefore, understanding the nonneuronal activities of DAF-16 is relevant for understanding life-span regulation.

Second, because our reporters were driven by upstream DNA sequences, we did not query potential regulatory sequences within introns or coding sequences of DAF-16-regulated genes. This is a concern, as the one translational fusion we did examine, from *mtl-1*, was highly *daf-2* responsive, whereas a transcriptional fusion to the same promoter sequence did not respond as well. In addition, we note that DAF-16 could potentially influence gene expression at the level of translation (McColl et al., 2010), and this would not be assessed in our study.

Finally, important *daf-2*/*daf-16*-regulated genes with small induction ratios would probably have escaped detection in our assay—and we could have missed, simply by chance, key life-span genes with properties that differ from the genes we examined. Nevertheless, our analysis of this small gene set suggests some interesting new features of this regulatory network.

Three Modes of DAF-16 Gene Regulation

Together, our findings provide molecular support for the idea that DAF-16 influences gene expression among the tissues of *C. elegans* in three ways (Figure 6E). First, DAF-16 can act within a tissue to regulate genes predicted to influence the health and longevity of that tissue. Second, DAF-16 activates downstream signal transduction cascades that act independently of *daf-16* to regulate gene expression at a distance and to slow the aging of other tissues (Libina et al., 2003) (this study) (FOXO-to-FOXO(−) signaling). Third, as shown previously, DAF-16 regulates the expression of insulin-like genes (Murphy et al., 2003), allowing DAF-16 activity in one tissue to affect DAF-16 activity elsewhere in the animal (FOXO-to-FOXO signaling) (Murphy et al., 2007).

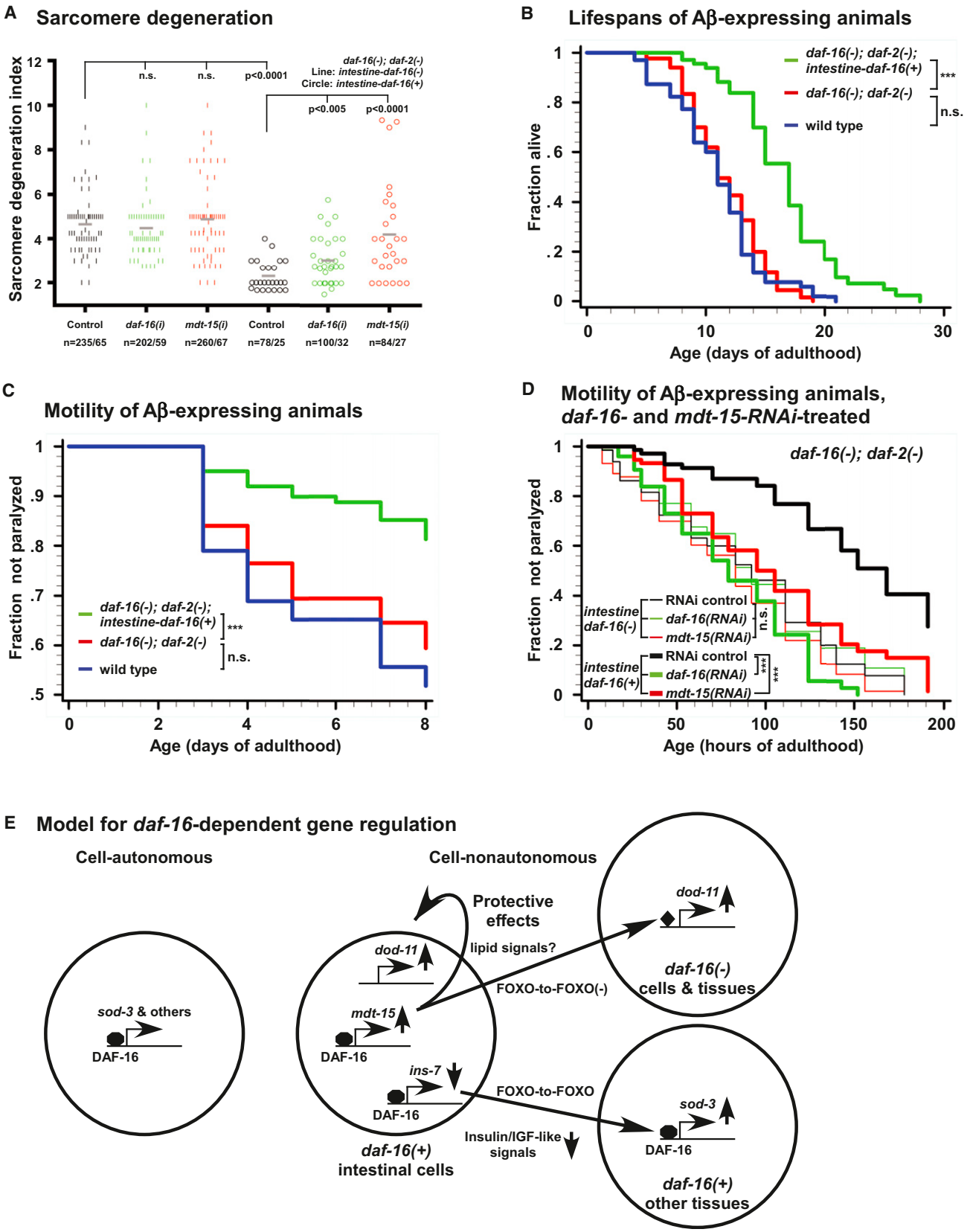
Cell-Autonomous Gene Regulation by DAF-16

Six of the nine *daf-2*/*daf-16*-responsive genes we examined were regulated in a strictly cell-autonomous fashion by DAF-16, as was the previously analyzed DAF-16-regulated gene *sod-3*. This finding argues against the model that DAF-16 directly regulates only downstream signaling genes, whose effects on other cells are completely responsible for life extension. Instead, the behavior of this gene sample, which includes genes with diverse functions, suggests that DAF-16 may activate numerous types of cell-protective genes cell autonomously. All but one promoter we analyzed contained canonical DAF-16-binding elements. The presence of DBEs suggests direct DAF-16 regulation, though our findings show that DBE-containing promoters, such as the *dod-11* and *hsp-12.6* promoters, can also be switched on by DAF-16 indirectly, through DAF-16's activity in other tissues. Conversely, DAF-16 could also act via a promoter fragment that lacked canonical DBEs, apparently by binding to suboptimal DBE sites, to the DAE/GATA site, and to other, unidentified sequences. FOXO proteins possess chromatin-remodeling ability (Hatta and Cirillo, 2007), so DAF-16 may utilize multiple binding sites to create a permissive environment for gene expression.

All but one of the twenty *daf-2*-dependent transgenes we examined (plus the control *sod-3* transgene) were expressed in the intestine, and eleven were expressed mainly or exclusively in the digestive tract. This intestinal enrichment was highly significant statistically ($p = 1.1E-11$; Table S1). Why might DAF-16 regulate so many intestinal genes? First, the intestine seems to be particularly vulnerable to aging, undergoing extensive tearing and deterioration (McGee et al., 2011). It is also a major entry port for toxins and bacterial pathogens, and becomes packed with bacteria with age (Garigan et al., 2002; McGee et al., 2011). Bacterial packing and intestinal deterioration are both reduced greatly by *daf-2* mutations (Garigan et al., 2002; McGee et al., 2011). Thus, DAF-16 may extend life and promote stress resistance in part by "bullet-proofing" the intestine. For instance, the intestinal metallothionein *mtl-1* may protect the animal from heavy metals it ingests. Finally, our 23 DAF-16-regulated, intestine-expressed genes include at least 15 metabolic genes (Table S1), raising the possibility that DAF-16 could act in the intestine to help nourish the animals to improve systemic health.

Insulin/IGF-1 signaling mutants are resistant to pathogenic bacteria (Evans et al., 2008; Garsin et al., 2003), possibly due to increased intestinal expression of the innate-immunity lysozyme gene *lys-7*. The intestine-specific GATA-factor ELT-2 may act with DAF-16 to protect the intestine from infection, as it is required for survival of wild-type animals exposed to pathogens, and for intestinal expression of *lys-7* (Figure 3A and Table 1) and other lysozymes (Shapira et al., 2006).

Finally, we note that, because intestinal DNA makes up only a small fraction of the animal's DNA, the intestinal enrichment of DAF-16-regulated genes could help explain why so few DAF-16-regulated genes from microarrays have been identified in chromatin profiling experiments (see Figure S4D, legend).


A
Sarcomere degeneration
Sarcomere degeneration index
n.s.
n.s.
p<0.0001
daf-16(-); daf-2(-)
Line: intestine-daf-16(-)
Circle: intestine-daf-16(+)
p<0.005
p<0.0001
Control
daf-16(i)
mdt-15(i)
Control
daf-16(i)
mdt-15(i)
n=235/65
n=202/59
n=260/67
n=78/25
n=100/32
n=84/27
B
Lifespans of Aβ-expressing animals
Fraction alive
daf-16(-); daf-2(-); intestine-daf-16(+)
daf-16(-); daf-2(-)
wild type
***
n.s.
Age (days of adulthood)
C
Motility of Aβ-expressing animals
Fraction not paralyzed
daf-16(-); daf-2(-); intestine-daf-16(+)
daf-16(-); daf-2(-)
wild type
***
n.s.
Age (days of adulthood)
D
Motility of Aβ-expressing animals, daf-16- and mdt-15-RNAi-treated
Fraction not paralyzed
daf-16(-); daf-2(-)
intestine daf-16(-)
RNAi control
daf-16(RNAi)
mdt-15(RNAi)
n.s.
intestine daf-16(+)
RNAi control
daf-16(RNAi)
mdt-15(RNAi)
***
***
Age (hours of adulthood)
E
Model for daf-16-dependent gene regulation
Cell-autonomous
Cell-nonautonomous
sod-3 & others
DAF-16
Protective effects
lipid signals?
dod-11
mdt-15
DAF-16
FOXO-to-FOXO(-)
dod-11
daf-16(-) cells & tissues
ins-7
DAF-16
FOXO-to-FOXO
sod-3
DAF-16
Insulin/IGF-like signals
daf-16(+) intestinal cells
daf-16(+) other tissues

GATA Factors Direct Expression of DAF-16-Regulated Genes to Specific Tissues

DAF-16 is expressed widely, and we found that DAF-16 activates genes tissue specifically, at least in part, by functioning in combination with tissue-specific GATA factors. The intestinal GATA-factor ELT-2 and the hypodermal factor ELT-3 were required for intestinal and hypodermal expression, respectively, of many DAF-16-regulated genes. Our findings, both in vivo and in vitro, suggest that GATA factors regulate these genes by binding to their DAE/GATA site (as predicted previously by McGhee et al., 2009, from DNA sequence). We note that some DAE-containing DAF-16-regulated genes, such as *lys-7* and *mtl-1*, are expressed exclusively in the intestine (Tables 1 and S1). Presumably, these genes contain additional sequences that prevent ELT-3 from activating them in the hypodermis. In addition, some DAF-16-regulated genes, like *tps-1*, were upregulated normally in both the intestine and the hypodermis in spite of GATA factor RNAi. Thus, DAF-16 does not absolutely require GATA factors to regulate gene expression in these tissues.

GATA Factors and Life Span

Since the intestinal GATA factor ELT-2 is needed for much intestinal DAF-16-regulated gene expression, it seemed likely that *elt-2* knockdown would shorten the life span of *daf-2(–)* mutants. We found that this was the case. Our data are consistent with a very recent, independent report showing that *elt-2* inactivation could disrupt cytoprotective gene expression and shorten the life span of *daf-2(–)* mutants (Shore et al., 2012). We found that *elt-2* knockdown also shortened the life span of calorically restricted *eat-2(–)* mutants substantially. This life-span pathway is *daf-16* independent but requires the FOXA transcription factor, *pha-4* (Panowski et al., 2007). Interestingly, *pha-4* expression is activated by ELT-2, and the two proteins have been shown to coregulate specific genes (Ao-kye-Danso et al., 2008).

Our findings also point to an important role for a new tissue, the hypodermis, in life-span regulation by DAF-16, as hypodermal-only *daf-16* expression could extend the life span of *daf-16(–); daf-2(–)* mutants up to ~30%. Because ELT-3 was required for hypodermal expression of four DAF-16-regulated genes we examined, one might expect that knocking down ELT-3 expression would shorten the life span of *daf-2(–)* mutants. However, as observed by the McGhee group (Tonsaker et al., 2012), this was not the case. (Our findings differ from those from the Kim lab [Budovskaya et al., 2008] in some respects; please see Figure S5 for discussion.) This finding implies that DAF-16 regulates important hypodermal longevity genes independently of ELT-3.

DAF-16 Action at a Distance: FOXO-to-FOXO(–) Signaling

In this study, we extended the case for FOXO-to-FOXO(–) signaling from the organismal level to the level of individual tissues (aging muscles) and genes (specifically, two metabolic genes *dod-11* and *dod-8,* and one chaperone gene, *hsp-12.6*). These findings put the concept of FOXO-to-FOXO(–) signaling on solid molecular footing. DAF-16 action in the intestine, which can extend life span substantially (by 50%–70%), affected *dod-11, hsp-12.6*, and *dod-8* expression in multiple tissues. In addition, DAF-16 could act in other tissues to affect gene expression in the intestine and elsewhere. This latter finding can help to explain how, given the fragility of the intestine, *daf-2(–)* mutants can live 50% longer than wild-type if *daf-16* is expressed only in nonintestinal tissues (Libina et al., 2003). In that case, perhaps DAF-16 can act at a distance to protect the intestine.

How can DAF-16 promote signaling across tissues? Others reported that the gene *scl-1* was a candidate downstream signaling gene, but we were unable to confirm this in our studies (see Supplemental Discussion). However, we identified a new candidate, the DAF-16-regulated gene *mdt-15*. MDT-15 is a transcriptional mediator subunit that regulates genes involved in lipid metabolism, so it could potentially induce lipid signals that act across the tissues to affect life span. Loss of *mdt-15* reduces *dod-11* expression in many tissues, including several that do not appear to express *mdt-15.* Thus, MDT-15 appears to act on the sending end of an intercellular signaling pathway that is activated by DAF-16.

mdt-15(RNAi) animals are unhealthy. However, two findings suggest that *mdt-15* plays an important role in aging. First, loss of *mdt-15* accelerated age-dependent sarcomere deterioration in *daf-2(–)* animals expressing intestine-only *daf-16(+)*, but not in *daf-2(–)* animals that were also *daf-16(–)* (Figure 6). Second, *mdt-15* inhibition had a greater life-shortening effect on *daf-2(–)* mutants than it had on wild-type (Figure 5C). Rogers et al. (2011) recently reported similar, independent findings and also showed that *mdt-15* was required for life-span extension by inhibiting the translation factor *ifg-1*/eIF4G. It will be interesting to learn more about this potential downstream signaling pathway in the future.

FOXO-to-FOXO Signaling

DAF-16 can act at a distance to upregulate DAF-16 activity elsewhere in the animal (Murphy et al., 2007). There are many

Figure 6. Effects of Intestinal DAF-16 and MDT-15 on Muscle Aging and Age-Related Disease

(A) *mdt-15* RNAi reduced the ability of intestinal DAF-16 to protect *daf-16(–)* muscles during aging. Top: average sarcomere degeneration indexes of RNAi-treated animals on day 12 of adulthood. Each point represents one animal (three to four images were taken for each animal). Higher number (y axis) represents more severe sarcomere degeneration. Mean degeneration index is indicated by the gray bar (Mann-Whitney-Wilcoxon test; n.s., not significant).

(B and C) Intestinal DAF-16 attenuated the toxicity of muscle-expressed Aβ protein. In (B), Kaplan-Meier survival analysis of transgenic animals in which human Aβ(1-42) is expressed in body-wall muscles (see Table S3). Log-rank test, ***$p < 0.001$. n.s., not significant. In (C), age-dependent Aβ aggregation-induced paralysis of the animals shown in Figure 6B (see Table S3). Log-rank test, ***$p < 0.001$. n.s., not significant.

(D) *daf-16* or *mdt-15* RNAi abolished the ability of intestinal DAF-16 to ameliorate toxic Aβ aggregation-induced paralysis. RNAi was initiated at the L4 stage, and RNAi-treated animals were scored for paralysis at room temperature (see Table S3). Log-rank test, ***$p < 0.001$. n.s., not significant.

(E) Model for *daf-16*-dependent gene regulation. First, DAF-16 can act directly on its target genes in a cell-autonomous fashion. Second, DAF-16 activity in one tissue (e.g., intestine, muscle) can stimulate downstream signaling pathways that act on *daf-16(–)* cells to influence gene expression, aging, and protein-aggregation toxicity (FOXO-to-FOXO(–) signaling). Analysis of MDT-15 suggests that downstream lipid signals may play a role in this signaling. Third, DAF-16 action in one tissue can affect DAF-16 activity elsewhere, for example, by feedback regulation of insulin genes (FOXO-to-FOXO signaling).

situations in which inhibiting insulin or IGF-1 responsiveness in certain tissues extends life span. However, in the great majority of these cases, whether FOXO is required in other, wild-type tissues is not known. These examples include (1) the extension of life span caused by loss of *daf-2* activity in the ectoderm (skin, neurons) of *C. elegans* (Apfeld and Kenyon, 1998), as well as (2) the suppression of longevity caused by neuron-only *daf-2* expression (or intestine or neuron-only *age-1*/PI3K expression) in *daf-2* (or *age-1*) mutant worms (Iser et al., 2007; Wolkow et al., 2000). Likewise, it is not known whether the ability of neuronal *age-1(+)* to influence intestinal *hsp* gene expression in *C. elegans* (Iser et al., 2011) requires intestinal FOXO activity. In mice, brain-specific loss of the IGF-1 receptor (Kappeler et al., 2008) or downstream IRS genes (Taguchi et al., 2007) can increase life span. Activating FOXO or inhibiting insulin signaling in adipose tissue can extend life span in flies (Giannakou et al., 2004; Hwangbo et al., 2004) and mice (Blüher et al., 2003). At least in flies, this condition appears to trigger FOXO-to-FOXO signaling (Hwangbo et al., 2004), but whether it might trigger FOXO-to-FOXO(−) signaling as well is not known. It would be interesting to carry out these experiments in a *foxo(−)* background to determine whether FOXO is required in responding tissues. Finally, given the importance of the *C. elegans* intestine, as well as the hypodermis, in life-span regulation, it would be interesting to ask whether insulin/IGF-1-pathway members could send life-extending signals from the intestines or the skin of higher organisms.

DAF-16 Can Suppress Symptoms of Age-Related Disease from a Distance

Long-lived insulin/IGF-1 mutants are resistant to many age-related diseases, so we asked whether DAF-16 might act at a distance in a disease setting. We found that, in a process that requires *mdt-15*, endodermal DAF-16 can partially suppress the paralysis caused by expressing Aβ in the muscles. DAF-16 and MDT-15 action could potentially reduce the expression of Aβ in muscles, or they could decrease the accumulation of toxic Aβ oligomer species. This finding is important, as it raises the possibility that systemic, FOXO-dependent signals, perhaps beneficial lipid signals, might be able to slow the progression of Alzheimer's disease, and possibly other age-related diseases, in humans.

EXPERIMENTAL PROCEDURES

Microscopy

Transgenic animals were analyzed at the young-adult stage. For quantitative analysis, GFP fluorescence in the anterior quarter of the intestine was measured using the OpenLab software.

Gel-Shift Assays

Proteins were expressed and purified from bacteria. Radioactively labeled DNA oligos were mixed with proteins, resolved on a polyacrylamide gel, and subjected to autoradiography.

Life-Span Analysis

Lifespan assays were performed as described (Apfeld and Kenyon, 1998). RNAi was initiated at the young-adult stage. The prefertile period of adulthood was used as t = 0 for life-span analysis. STATA software (version 10.1) was used for statistical analysis.

Paralysis

Worms expressing human Aβ(1-42) in body-wall muscles were raised at 20°C and analyzed as adults. Worms that failed to move when touched with a platinum wire were scored as "paralyzed" (Link, 1995). To avoid mis-scoring, paralysis assays were terminated by day 8 of adulthood when wild-type animals begin to move more slowly. RNAi-treatment was initiated at the L4 stage, and young adults were scored at room temperature (~22.5°C), which accelerated paralysis and helped to distinguish Aβ paralysis from aging effects on motility.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, five tables, Supplemental Experimental Procedures, Supplemental Discussion, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2012.12.013.

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