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PDP-1 Links the TGF-β and IIS Pathways to Regulate Longevity, Development, and Metabolism

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PDP-1 Links the TGF-β and IIS Pathways to Regulate Longevity, Development, and Metabolism

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Abstract

The insulin/IGF-1 signaling (IIS) pathway is a conserved regulator of longevity, development, and metabolism. In Caenorhabditis elegans IIS involves activation of DAF-2 (insulin/IGF-1 receptor tyrosine kinase), AGE-1 (PI 3-kinase), and additional downstream serine/threonine kinases that ultimately phosphorylate and negatively regulate the single FOXO transcription factor homolog DAF-16. Phosphatases help to maintain cellular signaling homeostasis by counterbalancing kinase activity. However, few phosphatases have been identified that negatively regulate the IIS pathway. Here we identify and characterize pdp-1 as a novel negative modulator of the IIS pathway. We show that PDP-1 regulates multiple outputs of IIS such as longevity, fat storage, and dauer diapause. In addition, PDP-1 promotes DAF-16 nuclear localization and transcriptional activity. Interestingly, genetic epistasis analyses place PDP-1 in the DAF-7/TGF-β signaling pathway, at the level of the R-SMAD proteins DAF-14 and DAF-8. Further investigation into how a component of TGF-β signaling affects multiple outputs of IIS/DAF-16, revealed extensive crosstalk between these two well-conserved signaling pathways. We find that PDP-1 modulates the expression of several insulin genes that are likely to feed into the IIS pathway to regulate DAF-16 activity. Importantly, dysregulation of IIS and TGF-β signaling has been implicated in diseases such as Type 2 Diabetes, obesity, and cancer. Our results may provide a new perspective in understanding of the regulation of these pathways under normal conditions and in the context of disease.


Introduction

Insulin/IGF-1 signaling (IIS) is a conserved neuroendocrine pathway that regulates longevity, development and energy metabolism across phylogeny [1,2]. In the roundworm Caenorhabditis elegans (C. elegans), activation of the DAF-2 insulin/IGF-1 receptor tyrosine kinase initiates an AAP-1/AGE-1 PI 3-kinase signaling cascade involving the downstream serine/threonine kinases PDK-1, AKT-1, and AKT-2 [3–7]. Activated AKT-1 and AKT-2 phosphorylate DAF-16, the single Forkhead Box O (FOXO) family transcription factor homolog in C. elegans [8]. Phosphorylation of DAF-16 results in its inactivation and sequestration in the cytosol [9,10]. Under low signaling conditions, DAF-16 translocates into the nucleus, where it can transactivate/repress hundreds of target genes [9–13].

The dauer is an alternative survival stage that worms can enter upon poor environmental conditions such as crowding [14]. Mutations in the kinases upstream of DAF-16 such as daf-2, age-1, pdk-1, akt-1 and akt-2 result in an increase in lifespan, dauer formation, fat storage and/or stress resistance, and loss-of-function mutations in daf-16 completely suppress these phenotypes [15–18]. In addition to the IIS pathway, dauer formation in C. elegans is also regulated by the DAF-7/TGF-β-like signaling pathway [19–21]. Activation of TGF-β signaling is achieved through binding of the DAF-7 BMP-like ligand to the DAF-1/DAF-4, the Type I/II receptors, which phosphorylate and activate the downstream receptor-associated SMAD (R-SMAD) proteins DAF-8 and DAF-14, presumably through a conserved SSXS phosphorylation motif that has been shown to be important for R-SMAD activation in mammals [22–24]. Upon activation, R-SMADs can associate with a Co-SMAD to regulate the transcription of hundreds of genes [23,25]. In C. elegans, DAF-8 and DAF-14 act to antagonize the transcriptional activity of the DAF-3 Co-SMAD and the DAF-5 SNO-SKI repressor [22,24,26–29]. Reduction of function mutations in daf-7, daf-1, daf-4, daf-8 and daf-14 show temperaturesensitive constitutive dauer formation and mutations in daf-3 and/or daf-5 completely suppress this phenotype [19,21,30]. Genetic epistasis studies have suggested that the TGF-β pathway acts in a parallel manner with IIS to modulate dauer formation [31–33]. The PTEN lipid phosphatase homolog DAF-18, which antagonizes signaling at the level of AGE-1/PI 3-kinase, is a major negative regulator of IIS. In contrast to the kinases in this pathway, loss-of-function mutations in daf-18 reduces lifespan, fat storage, dauer formation and stress resistance [32,34–39]. Besides DAF-18, few negative modulators of the pathway have been identified. In particular, less is known about serine/threonine phosphatases that counterbalance kinase activity in the IIS pathway. We recently performed a directed RNA interference
Author Summary

Cells in the body respond to a variety of on/off signals that are relayed in a defined spatial and temporal manner. These signals influence several processes such as growth, fat storage, and the repair of damaged molecules. As humans age, the onset of diseases such as Type 2 Diabetes, obesity, and cancer often results from an imbalance in the levels of on/off signals in the cell. The insulin/IGF-1 signaling pathway is an important regulator of longevity, development, and metabolism across phylogeny. While the protein kinases that activate this pathway have been well studied, less is known about the protein phosphatases that tune down the signals. The roundworm C. elegans has been an excellent model system to study the role of insulin/IGF-1 signaling in the aging process. Here, we identify a new phosphatase that negatively regulates the insulin/IGF-1 pathway to enhance longevity and stress-resistance. Interestingly, the phosphatase achieves this function by tuning down the activity of a conserved TGF-β pathway, a pathway important for development. By reducing TGF-β pathway activity, this phosphatase decreases expression of insulin molecules that may stimulate the insulin/IGF-1 pathway. Our studies not only unravel a new regulator of these pathways, but also point to how they are more linked than previously thought. Both insulin/IGF-1 and TGF-β signaling have been implicated in age-associated diseases, and understanding their connection will provide us with potential therapeutic avenues.

Results

C. elegans PDP-1 regulatesdaf-2 dauer formation independent of PDH

Our RNAi screen was designed to identify serine/threonine phosphatases that regulate C. elegans IIS using dauer formation as an output [39]. We identified the P2A regulatory subunit PPT1R-1 as an important regulator of AKT-1 dephosphorylation as well as DAF-16-dependent phenotypes [39]. Here we characterize another candidate from this screen, pdp-1, as a positive regulator of dauer formation. PDP-1 is homologous to pyruvate dehydrogenase phosphatase (PD-P) in higher organisms, an enzyme that positively regulates the pyruvate dehydrogenase enzyme complex (PDHC). RNAi of the other components of PDHC do not result in changes in dauer formation. Interestingly, we report that although PDP-1 is a robust modulator of multiple IIS-regulated processes as well as DAF-16 activity, genetic epistasis studies place pdp-1 in the DAF-2/TGF-β pathway. Through this study, we find that IIS and TGF-β signaling are more tightly connected than previously suggested, with distinct roles for the Co-SMAD DAF-3 in modulating the IIS pathway. Our data suggests that PDP-1 modulates the gene expression of several insulins, and that insulins may be a potential mediator of the crosstalk between these two pathways.

mammalian PDP (~52% positive and ~38% identical). pdp-1 RNAi significantly reduces dauer formation of daf-2(e1370) worms, similar to daf-18 RNAi (Figure 1A and Figure S2). This phenotype is not allele-specific, as pdp-1 RNAi results in suppression of dauer formation in a second allele of daf-2, daf-2(e1368) (Figure 1B and Figure S2). Similar to the results with the RNAi, a mutation in pdp-1 also affects dauer formation - pdp-1(m5734); daf-2(e1370) double mutants form significantly fewer dauers when compared to the daf-2(e1370) parental strain (Figure S2).

Given its homology to PD-P in higher organisms, we wondered whether the effect of pdp-1 knockdown on daf-2 dauer formation was a consequence of modulating the activity of the PDHC. The PDHC is a multi-subunit enzyme complex consisting of three major enzymes: E1 pyruvate dehydrogenase, E2 dihydrolipoyl acetyltransferase and E3 dihydrolipoyl dehydrogenase that regulate energy metabolism [40]. PDHC converts pyruvate to acetyl-coA, which can either enter the TCA cycle or be used for fatty acid synthesis. In mammals, regulation of PDHC activity is primarily achieved through reversible phosphorylation/dephosphorylation of the E1z subunit by pyruvate dehydrogenase kinase (PDHK) and PD-P, with phosphorylation inactivating the enzyme complex [40]. All of the components of the PDHC complex have conserved C. elegans homologs, encoded by the genes T05H11.06 (E1z), C04C3.3 (E1β), F23B12.5 (E2), LLC1.3 (E3), pdhk-2 (PDHK) and pdp-1 (PD-P).

To test whether modulation of PDHC activity affects daf-2 dauer formation, we grew daf-2(e1370) worms on PDHC RNAi. Quantification the RNAi efficiency of the PDHC components revealed that we achieved 60–90% knockdown (Figure S1). To our surprise, RNAi of the E1z subunit had no effect on daf-2 dauer formation, while pdp-1 RNAi resulted in dauer suppression (Figure 1C and Figure S2). In addition, RNAi of either the other E1 subunit E1β, or the E2 subunit, did not affect daf-2 dauer formation (Figure 1C and Figure S2). Knockdown of the E3 subunit resulted in lethality (data not shown). Interestingly, pdhk-2 RNAi resulted in slight suppression daf-2(e1370) dauer formation but had no effect on dauer formation of daf-2(e1368) mutants (Figure 1C and Figure S2). Therefore pdhk-2 modulates the IIS pathway in an allele-specific manner and we did not perform further characterization of this gene.

To further evaluate the components of the PDHC complex, we examined their expression patterns. The expression pattern of PD-P does not completely overlap with that of the E1 and E2 subunits of PDHC (Figure S3). PD-P expression was enriched in the head and tail neurons, head muscle and the intestine. We did not observe any expression in the pharynx. In contrast, the expression of the E1 and E2 subunits, was observed throughout the body of the worm and was significantly enriched in the pharynx. Taken together, PDP-1 modulates daf-2 dauer formation and this function is likely to be independent of its role in regulating the PDHCs.

PDP-1 regulates multiple outputs of the IIS pathway

In addition to dauer formation, the IIS pathway also regulates longevity, stress resistance and fat storage [17,18]. Mutations in daf-2 and age-1 result in a significant extension in lifespan, enhanced resistance to various stresses and increased fat storage [7,35,41–44]. These phenotypes are suppressed by loss-of-function mutations in daf-18 and daf-16 [32,34,35,39]. We therefore investigated whether dosage modulation of pdp-1 would affect additional outputs of the pathway. We first tested the role of PDP-1 in regulating lifespan (Figure 2 and Figure S4). The lifespan of wild-type worms was not affected by pdp-1 RNAi and slightly reduced by a mutation in pdp-1 (Figure 2A and 2D). In contrast, the mean and maximal lifespan of long-lived daf-2(e1370) and age-
1(hx546) mutants was significantly reduced by pdp-1 RNAi (Figure 2B and 2C). Similarly, pdp-1(tm3734); daf-2(e1370) double mutants lived significantly shorter than the parental daf-2(e1370) strain (Figure S4).

To examine the effect of increased dosage of pdp-1, we generated transgenic worms bearing a translational fusion containing pdp-1 fused to gfp and driven by its own promoter (pdp-1::gfp). In addition, we also crossed the pdp-1::gfp worms to daf-2(e1370) mutants to generate the daf-2(e1370); pdp-1::gfp strain. Overexpression of pdp-1 results in a significant extension in lifespan compared to wild-type worms (Figure 2D and Figure S4). Interestingly, pdp-1 overexpression further extends the lifespan of daf-2(e1370) mutants (Figure 2B and Figure S4). In both of these cases, the increased lifespan was suppressed by daf-16 RNAi (Figure S5). Therefore, dosage modulation of pdp-1 regulates lifespan in a DAF-16 dependent manner.

Next, we asked if PDP-1 modulated additional outputs of the IIS signaling pathway. We first tested whether PDP-1 regulates stress resistance by assaying the survival of pdp-1 mutants and transgenic animals when exposed to heat stress at 37°C (Figure 2E and Figure S7). Dosage modulation of pdp-1 affects the response to heat stress, with a pdp-1 mutation decreasing and pdp-1 overexpression slightly enhancing thermotolerance (Figure 2E). Importantly a pdp-1 mutation drastically reduced the thermotolerance of daf-2 mutants (Figure 2E).

To examine the role of pdp-1 in regulating fat storage, we used both Oil Red O [45] and Sudan Black [7] staining (Figure 2F and 2G and Figure S7). pdp-1 mutants had similar levels of fat compared to wild-type worms, while overexpression of pdp-1 slightly enhanced fat storage (Figure S7). In contrast, a pdp-1 mutation drastically reduced the increased fat of daf-2(e1370) mutants (Figure 2F and 2G and Figure S7). This was observed in dauers, larval stage 3 (L3) animals and adults, suggesting that PDP-1 is an important regulator of fat storage in daf-2 mutants.

We did observe any further enhancement of the increased fat storage in the daf-2(e1370); pdp-1::gfp worms (Figure S7). Importantly, the increased fat storage of pdp-1::gfp and daf-2(e1370); pdp-1::gfp worms was suppressed by daf-16 RNAi, similar to daf-2 mutants (Figure S7). Thus, PDP-1 modulates all four well-characterized outputs of the IIS pathway.
In addition to these phenotypes, pdp-1(tm3734) mutants exhibit a slow movement phenotype, which we quantified using locomotion assays (Figure S6). This slow movement was rescued by the pdp-1::gfp transgene. In addition, we performed brood size analysis of wild-type, pdp-1(tm3734) mutants, daf-2(e1370) mutants, and pdp-1(tm3734); daf-2(e1370) double mutants (Figure S6). pdp-1(tm3734) worms showed a slight decrease in the number of progeny compared to wild-type worms. However, when compared to daf-2 mutants, only 5% of the pdp-1(tm3734); daf-2(e1370) eggs yielded progeny (Figure S6). daf-2 mutants have a slightly reduced brood size [46,47], and a mutation in pdp-1 severely enhances this phenotype. Taken together, PDP-1 regulates multiple outputs of IIS and acts as a negative regulator the pathway, similar to DAF-18/PTEN.

PDP-1 positively regulates DAF-16

The FOXO transcription factor DAF-16 is the major target of the C. elegans IIS pathway [2,48]. Under conditions of reduced IIS, DAF-16 is able to translocate into the nucleus, where it regulates the expression of hundreds target genes [12,13,49,50]. We therefore asked whether PDP-1 modulates DAF-16 subcellular localization as well as activity (Figure 3A and Figure S8). daf-2(e1370); pdp-1::gfp worms were grown on vector, daf-18 and pdp-1 RNAi, and DAF-16 nuclear/cytoplasmic localization was visualized using fluorescence microscopy and quantified. Throughout the body of the worm, while DAF-16::GFP was mostly nuclear on vector RNAi, its localization was enriched in the cytosol on pdp-1 RNAi, similar to daf-18 RNAi (Figure 3A and Figure S8).

Figure 2. PDP-1 regulates multiple outputs of the IIS pathway. Data shown are from one representative experiment. (A) pdp-1 RNAi does not significantly reduce the lifespan of wild-type worms. Mean lifespan of wild-type worms is 23.8 ± 6 days (n = 93) on vector RNAi, 14.5 ± 9 days (n = 34) on daf-18 RNAi (p < 0.0001) and 22.6 ± 0.6 days (n = 68) on pdp-1 RNAi (p < 0.08). (B) The increased lifespan of daf-2(e1370) worms is reduced by pdp-1 RNAI. Mean lifespan of daf-2(e1370) worms is 38.9 ± 0.9 days (n = 75) on vector RNAI, 24.5 ± 0.5 days (n = 59) on daf-18 RNAI (p < 0.0001) and 31.7 ± 0.8 days (n = 66) on pdp-1 RNAI (p < 0.0001). (C) pdp-1 RNAI reduces the increased lifespan of age-1(tm546) mutants. Mean lifespan of daf-2(e1370) worms is 42.8 ± 0.8 days (n = 84) on vector RNAI, 28.0 ± 0.9 days (n = 81) on daf-18 RNAI (p < 0.0001) and 36.5 ± 1.0 days (n = 67) on pdp-1 RNAI (p < 0.0001). (D) pdp-1 overexpression increases the lifespan of wild-type and daf-2(e1370) worms while pdp-1 mutants live slightly shorter than wild-type animals. Mean lifespan of wild-type worms is 29.4 ± 0.5 days (n = 104), pdp-1(tm3734) mutants was 27.1 ± 0.7 days (n = 98), p < 0.05, pdp-1::gfp mutants is 34.5 ± 0.8 days (n = 92) p < 0.0001, daf-2(e1370) is 38.7 ± 0.7 days (n = 108) and daf-2(e1370); pdp-1::gfp is 42.8 ± 0.7 days (n = 105) days p < 0.0001. (E) PDP-1 regulates thermotolerance. Mean survival of wild-type worms is 18.3 ± 0.7 hours (n = 37), p < 0.0001) and 17.1 ± 0.8 hours (n = 27) < 0.02, pdp-1::gfp worms is 19.7 ± 0.9 days (n = 25) p < 0.09, daf-2(e1370) worms is 21.6 ± 0.6 hours (n = 30) and pdp-1(tm3734); daf-2(e1370) worms is 18.6 ± 0.9 hours (n = 19), < 0.0007). (F) Oil Red O staining reveals that pdp-1::gfp reduces the increased lifespan of wild-type worms while pdp-1(e1370) fat storage in both, L3s (p < 0.0001) and young adults (p < 0.01). (G) Oil Red O staining reveals that pdp-1::gfp worms store slightly more fat than daf-2(e1370) not in younger L3 animals (p < 0.02).
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The gene superoxide dismutase 3 (sod-3) is a direct DAF-16 target [11]. To test whether PDP-1 modulates transcriptional activity of DAF-16, we used a *Psod-3::gfp* reporter strain in a *daf-2(e1370)* background [51]. *daf-2(e1370); Psod-3::gfp* worms were grown on vector, *pdp-1, daf-18* and *daf-16* RNAi and GFP expression was visualized using fluorescence microscopy and scored as low, medium or high (Figure 3B and Figure S8). GFP expression was markedly lower on *pdp-1* RNAi compared to vector RNAi, similar to *daf-18* and *daf-16* RNAi, suggesting that PDP-1 positively modulates DAF-16 transcriptional activity. To further validate these results, we used quantitative real-time PCR (Q-PCR) to look at the expression levels of well-known DAF-16 target genes [52] in *daf-2(e1370), pdp-1(tm3734), daf-2(e1370)* and *daf-16(mgDf50) worms* (Figure 3C). Notably, the expression of *sod-3, sod-5* and *hsp-12.6* was significantly reduced in *pdp-1(tm3734); daf-2(e1370)* mutants relative to *daf-2(e1370)*. Therefore PDP-1 positively regulates a subset of DAF-16 targets.

**PDP-1 acts in the DAF-7/TGF-β signaling pathway**

Thus far our data indicates that PDP-1 regulates multiple outputs of IIS as well as DAF-16 activity. Using dauer formation as the readout, we performed genetic epistasis experiments to identify the substrate of PDP-1. We first tested whether *pdp-1* acted directly through the IIS pathway by focusing on kinase mutants downstream of *daf-2* (Table 1 and Figure S9). *pdk-1(sa680), daf-2(e1370); akt-1(ok525)* and *daf-2(e1370); akt-2(ok393)* mutants were maintained on vector RNAi, *daf-18* and *pdp-1* RNAi compared to vector RNAi, similar to *daf-18* and *daf-16* RNAi, suggesting that PDP-1 positively modulates DAF-16 transcriptional activity. To further validate these results, we used quantitative real-time PCR (Q-PCR) to look at the expression levels of well-known DAF-16 target genes [52] in *daf-2(e1370), pdp-1(tm3734), daf-2(e1370)* and *daf-16(mgDf50)*; *daf-2(e1370)* worms (Figure 3C). Notably, the expression of *sod-3, sod-5* and *hsp-12.6* was significantly reduced in *pdp-1(tm3734); daf-2(e1370)* mutants relative to *daf-2(e1370)*. Therefore PDP-1 positively regulates a subset of DAF-16 targets.

We next examined a TGF-β pathway that also regulates dauer formation [19–21] using genetic epistasis analyses with mutants of this pathway. In these assays, TGF-β pathway mutants were maintained on vector RNAi, *pdp-1* RNAi and *daf-3* RNAi (as a positive control; Table 2 and Figure S10). We first tested *daf-7* mutants, which contain a mutation in the gene encoding the TGF-β ligand [53]. Dauer formation of *daf-7(e1372)* mutants was suppressed on *pdp-1* RNAi similar to *daf-3* RNAi, suggesting that *pdp-1* does not function at the level of *daf-7* (Table 2 and Figure S10). Therefore PDP-1 links TGF-β and IIS signaling.

**Figure 3. PDP-1 regulates DAF-16 nuclear localization and transcriptional activity.** (A) DAF-16::GFP localization visualized in *daf-2(e1370); daf-16::gfp* worms on vector, *daf-18* and *pdp-1* RNAi (top panel, 100× magnification) and quantification of DAF-16::GFP nuclear-cytosolic localization (lower panel). Data shown are from one representative experiment. (n = 68 on vector RNAi, n = 88 on *daf-18* RNAi and n = 79 on *pdp-1* RNAi). (B) Representative images of high, medium and low GFP expression in *daf-2(e1370); Psod-3::gfp* worms (top panel, 100× magnification). Quantification of GFP expression in *daf-2(e1370); Psod-3::gfp* worms on vector, *daf-18, pdp-1* and *daf-16* RNAi (Lower panel). Data shown are from one representative experiment (n = 31 on vector RNAi, n = 27 on *daf-18* RNAi and n = 28 on *daf-16* RNAi). (C) Levels of known DAF-16 targets are reduced in *pdp-1(tm3734); daf-2(e1370)* worms when compared to *daf-2(e1370)* worms. Data shown is an average of three independent repeats. * p<0.05, **p<0.01. doi:10.1371/journal.pgen.1001377.g003
Next, we tested dauer formation with mutants of the SMADS daf-8 and daf-14 [22]. We grew daf-14(m77) mutants on vector, pdp-1 and daf-3 RNAi. Interestingly, pdp-1 RNAi had no effect on daf-14 dauer formation, while daf-3 RNAi still resulted in suppression (Table 2 and Figure S10). We next looked at dauer formation of daf-8(m85) mutants and again observed that pdp-1 RNAi had no effect, while daf-3 RNAi suppressed dauer formation (Table 2 and Figure S10). Therefore, our genetic epistasis results indicate a genetic interaction between pdp-1 and daf-14/daf-8.

To confirm these results, we investigated whether pdp-1 RNAi could suppress dauer formation of daf-2(e1370); daf-3(mgD90) double mutants (Table 2 and Figure S10). In this strain, input from the TGF-β pathway is removed due to the daf-3 null mutation, and dauer formation is presumably mediated through activated DAF-16 [39,40]. Therefore, if pdp-1 was indeed acting in the TGF-β pathway, we would not see any effect of pdp-1 RNAi on daf-2(e1370); daf-3(mgD90) double mutants. Expectedly, pdp-1 RNAi had no effect on daf-2(e1370); daf-3(mgD90) double mutants (Table 2 and Figure S10). DAF-3 itself is unlikely to be a substrate for PDP-1, as similar to mammalian Co-SMADs, it lacks the SMAD phosphorylation motif [29]. Therefore, our genetic epistasis analysis supports a model whereby pdp-1 acts in the DAF-7 TGF-β pathway at the level of daf-8 and daf-14.

**Table 1. Genetic epistasis analysis using IIS mutants.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>vector RNAi</th>
<th>daf-18 RNAi</th>
<th>pdp-1 RNAi</th>
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<tbody>
<tr>
<td>pdk-1(isa680)</td>
<td>85.0 ± 4.7 (520)</td>
<td>35.3 ± 2.5 (327)*</td>
<td></td>
</tr>
<tr>
<td>daf-2(e1370)</td>
<td>8.3 ± 3.6 (476)</td>
<td>5.3 ± 1.0 (241)</td>
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</tr>
<tr>
<td>daf-1(e1370); akt-1(ok935)</td>
<td>36.9 ± 1.4 (390)</td>
<td>35.9 ± 0.9 (265)*</td>
<td>16.0 ± 0.4 (375)*</td>
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<tr>
<td>daf-2(e1370)</td>
<td>75.6 ± 4.8 (247)</td>
<td>0.3 ± 1.0 (777)*</td>
<td>17.3 ± 8.2 (597)**</td>
</tr>
<tr>
<td>daf-3(mgD90)</td>
<td>61.1 ± 15.3 (289)</td>
<td>4.1 ± 1.7 (308)**</td>
<td>11.5 ± 3.6 (301)**</td>
</tr>
</tbody>
</table>

Assays were performed at 22.5°C, 19.2°C and 20°C.

**Table 2. Genetic epistasis analysis using TGF-β signaling mutants.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>vector RNAi</th>
<th>daf-3 RNAi</th>
<th>pdp-1 RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>daf-7(e1372)</td>
<td>85.3 ± 1.1 (612)</td>
<td>43.4 ± 0.8 (134)**</td>
<td>32.2 ± 4.9 (122)*</td>
</tr>
<tr>
<td>daf-14(m77)</td>
<td>81.7 ± 5.6 (543)</td>
<td>18.1 ± 8.9 (441)**</td>
<td>88.7 ± 1.3 (335)</td>
</tr>
<tr>
<td>daf-8(e1365)</td>
<td>32.0 ± 9.7 (392)</td>
<td>2.3 ± 1.8 (396)**</td>
<td>34.6 ± 9.1 (430)</td>
</tr>
<tr>
<td>daf-2(e1370); daf-3(mgD90)</td>
<td>50.8 ± 0.4 (302)</td>
<td>49.5 ± 2.5 (270)</td>
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</table>

Assays were performed at 22.5°C, 20°C and 19.2°C. *p<0.01. **p<0.05.

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A number of studies have previously identified roles for the TGF-β pathway in lifespan and fat storage [7,54,55]. However, genetic epistasis analysis on dauer formation placed DAF-7 TGF-β signaling and IIS as two parallel pathways where components of one pathway did not affect the other [14,56,57]. Yet in our studies, PDP-1 was able to regulate multiple outputs of IIS. Therefore, we decided to further investigate the potential crosstalk between the IIS and TGF-β signaling pathways. First, we focused on DAF-3 and DAF-5, which are positive regulators of dauer formation similar to PDP-1, and asked whether mutations in daf-3 or daf-5 could also affect phenotypes of the IIS pathway [14,28,29].

We tested lifespan, fat storage, dauer formation and stress resistance of TGF-β pathway mutants in a wild-type as well as daf-2(e1370) background. (Figure 4A–4C, Figure S11, S12, S13 and Table S1). As previously reported, the lifespan of daf-3 and daf-5 single mutants is slightly shorter than wild-type worms (Table S1) [55]. In our hands, mutations in the upstream components of the TGF-β pathway such as daf-7 and daf-14 enhance dauer formation but do not significantly extend lifespan (Table S1 and Figure S4). Intriguingly, mutations in daf-3 and daf-5 have opposite effects on daf-2(e1370) phenotypes. When compared to the daf-2(e1370) parental strain, daf-2(e1370); daf-3(mgD90) mutants lived significantly longer. This was also observed in daf-2(e1370); daf-16 worms, which is a weaker allele of daf-3. In contrast, daf-5(e1386); daf-2(e1370) double mutants live much shorter than daf-2(e1370) worms (Figure 4A, Figure S13 and Table S1). A mutation in daf-5 also decreased the increased lifespan of age-1(hx546) worms, with age-1(hx546); daf-5(e1385) double mutants living significantly shorter than the parental strain (Figure S13). Importantly, for daf-2 worms, the effect of a daf-3 null mutation on lifespan was more pronounced at 20°C where signaling through the IIS pathway is further reduced. Therefore, under low IIS conditions, DAF-3 as well as DAF-5 can modulate longevity.

We next tested the role of DAF-3 and DAF-5 on fat storage, dauer formation and stress resistance. Oil Red O staining for fat showed comparable levels between daf-2(e1370) and daf-2(e1370); daf-3(mgD90) worms, but markedly lesser amounts of fat in daf-5(e1386); daf-2(e1370) worms (Figure 4B top and bottom panel and Figure S12). Similarly, age-1(hx546); daf-5(e1385) had less fat than age-1(hx546) worms (Figure S12). Both daf-3 and daf-5 single mutants have slightly reduced levels of fat when compared to wild-type worms (Figure S12).

A similar trend was seen with our data for dauer formation. daf-2(e1370); daf-3(mgD90) worms show significant enhancement of daf-2(e1370) dauer formation across several temperatures tested, whereas a daf-3 mutation or daf-5 RNAi results in reduced daf-2(e1370) dauer formation (Figure 4C, Figure 4Cii and Figure S11). In addition, daf-5(e1386); daf-2(e1370) worms fail to completely arrest at the restrictive temperature of 25°C (data not shown). A mutation in daf-5 also significantly reduces thermotolerance of daf-2(e1370) worms at 37°C (Figure S13). Taken together, similar to PDP-1, DAF-3 and DAF-5 modulate multiple outputs of the IIS pathway. Unexpectedly, we find that while DAF-3 promotes dauer formation under conditions of reduced TGF-β signaling, it negatively regulates dauer formation and longevity under conditions of reduced IIS.

To further explore the crosstalk between both pathways, we next asked whether DAF-18 and DAF-16, which are components of the IIS pathway, affect TGF-β pathway signaling. For this, we assayed dauer formation of TGF-β pathway mutants on daf-18 and daf-16 RNAi (Table 3 and Figure S10). Interestingly, dauer formation of daf-7(e1372), daf-14(m77) and daf-8(m85) worms was robustly suppressed by daf-16 RNAi. We observed similar results for dauer formation of daf-7(e1372) and daf-14(m77) mutants on daf-
18 RNAi. However, in the case of daf-8(m85) mutants, daf-18 RNAi had no effect on dauer formation of (Figure S10), suggesting a complex crosstalk between both pathways. The enhanced dauer formation of daf-2(e1370); daf-3(mgDf90) is suppressed by both daf-18 and daf-16 RNAi but not pdp-1 RNAi (Table 3 and Figure S10). Therefore, we not only observe DAF-3 and DAF-5 affecting various phenotypes of the IIS pathway, but also the converse, where DAF-16 and DAF-18 robustly regulates TGF-β dauer formation. These results unravel a more complex interaction between the two pathways, where DAF-16 is likely to be the major downstream effector regulating longevity, dauer formation and other physiological outputs.

Insulins are a possible connection between TGF-β signaling and IIS

How can these two pathways, once considered to be parallel to each other, be mechanistically linked? Thus far our data suggests that PDP-1, a component of the TGF-β pathway can modulate multiple phenotypes of IIS by positively regulating DAF-16. In addition, we observe extensive crosstalk between the two pathways.
at multiple levels. A feed-forward model that has been proposed to connect TGF-β signaling to the IIS pathway suggests insulins as a possible link [55,58]. The C. elegans genome encodes 40 insulin genes [59,60] (WormBase 215: www.wormbase.org). Studies using mutants and RNAi have characterized some of the insulins as agonists or antagonists of the IIS pathway [13,59–61]. Importantly, microarray studies have identified several insulin genes that are regulated by TGF-β signaling, including ins-1, ins-4, ins-5, ins-6, ins-7, ins-17, ins-18, ins-30, ins-33, ins-35 and daf-28 [55,57]. We tested changes in the levels of these insulins using Q-PCR in TGF-β pathway mutants such as daf-3(mgDf90), daf-14(m77) as well as pdp-1(tm3734) and compared them to wild-type worms (Figure 5A–1(tm3734), Figure S14, Tables S2 and S3). Interestingly, both pdp-1(tm3734) and daf-3(mgDf90) showed elevated levels of several insulins as compared to wild-type worms (Figure 5A and Figure S14). In contrast, expression of these insulins was markedly reduced in daf-14(m77) mutants (Figure 3B and Figure S14). We next looked at the effects of overexpressing DAF-3 and PDP-1 on insulin gene expression (Figure 5C and Figure S14). The levels of several insulins are markedly reduced in daf-3 and daf-14 mutants. Moreover, dosage modulation of DAF-3 and PDP-1 modulates insulin gene expression. INS-4, for example, has been reported as a positive modulator of DAF-3 and PDP-1 modulates insulin gene expression. Therefore, PDP-1 would modulate to regulate expression of several insulins that can potentially feed into or antagonize the IIS pathway to regulate DAF-16 and its associated phenotypes.

**Discussion**

We identified pdp-1 from a RNAi screen for serine/threonine phosphatases that modulate daf-2 dauer formation. C. elegans PDP-1 is homologous to mammalian pyruvate dehydrogenase phosphatase (PDP), a metabolic enzyme that is a positive regulator of the pyruvate dehydrogenase enzyme complex (PDEc). Remarkably, other components of the PDEc in C. elegans do not affect dauer dauer formation. Microarray and SAGE studies on dauer have indicated that genes involved in anaerobic metabolism are upregulated while genes involved in the TCA cycle and mitochondrial oxidative phosphorylation are downregulated, suggesting that PDEc activity may not be critical for dauer diapause [64–66]. Further, annotations indicate that the C. elegans genome encodes approximately 60 serine/threonine phosphatases, in contrast to the 400 plus protein kinases, suggesting that phosphatases are likely to have a number of cellular substrates [39,67]. We find that PDP-1 also regulates longevity, fat storage and stress resistance in addition to dauer formation. Interestingly, these phenotypes are more severe in mutants such as daf-2 and age-1, where IIS is reduced. Further, PDP-1 positively regulates DAF-16 activity. We reason that PDP-1 function is critical under conditions of stress or low food availability, when DAF-16 activation is required [39].

Intriguingly, genetic epistasis analyses place PDP-1 in the DAF-7/TGF-β pathway, at the level of the R-SMAD proteins DAF-14 and DAF-8. A recent functional RNAi screen for serine/threonine phosphatases that modulate BMP signaling identified PDP as a SMAD1 phosphatase in Drosophila S2 cells and mammalian 293T cells [68]. Our study complements these findings and reveals a molecular conservation in the role of PDP-1 in regulating TGF-β signaling. Early genetic epistasis studies had suggested that TGF-β signaling and IIS pathways are parallel signaling pathways that modulate dauer diapause [31]. Importantly, in these studies, the conclusion was that both these pathways acted independently, and it was the IIS pathway that regulated longevity and stress resistance [31,32].

However, the effect of PDP-1 on DAF-16 activity led us to re-investigate the interaction between the IIS and TGF-β signaling. Previous studies have shown that DAF-3 and DAF-5 are negatively regulated by TGF-β signaling, and function similarly as repressors of gene expression to ultimately promote dauer formation [28,29,69,70]. We find that under conditions of reduced IIS, DAF-3 and DAF-5 affect various outputs of the IIS pathway.
in opposite ways. DAF-3 in particular regulates IIS depending upon the level of signaling through the pathway (Figure 6). In our hands, mutants of the TGF-β signaling pathway do not exhibit a pronounced increase in lifespan. However, components of this pathway are important for the long lifespan of mutants in the IIS pathway, as well as other phenotypes such as dauer formation, fat storage and stress resistance. Our epistasis studies reveal that daf-18 and daf-16 RNAi can strongly suppress dauer and fat storage of TGF-β pathway mutants. Together, these results point to a feed-forward model where signals through the TGF-β pathway are relayed to modulate activity of the IIS pathway as well as DAF-16. Indeed, recent studies have suggested that TGF-β pathway regulates the expression of insulins, leading to a feed-forward model, where signals from the TGF-β pathway are relayed to modulate activity of the IIS pathway as well as DAF-16 [55,58].

In support of this model, we find TGF-β signaling regulates the expression of several insulin genes with DAF-3 and PDP-1 negatively modulating insulin gene expression. This is in agreement with previous studies that identify DAF-3 as a repressor of gene expression [69,70]. The expression of several insulins is also modulated by DAF-16, with pdp-1(tm3734); daf-2(e1370) and daf-16(mgDf50); daf-2(e1370) worms showing similar trends in insulin levels. Therefore, in the absence of PDP-1, increased levels of agonists or reduced levels of antagonists hyperactivate the DAF-2 pathway to negatively regulate DAF-16, thereby affecting the enhanced lifespan, stress resistance, dauer formation and fat storage of daf-2 mutants.

Our results suggest a model where under favorable growth conditions, signals through the TGF-β pathway activate the SMAD transcriptional complex to regulate the expression of insulins that activate the IIS pathway to phosphorylate and inhibit DAF-16 activity, thereby promoting growth, reproduction and normal lifespan (Figure 6, top panel). However, when food is limiting or under harsh survival conditions, TGF-β signaling is downregulated by PDP-1 to activate DAF-3 and DAF-5, to regulate the repression of insulin genes that may feed into the IIS pathway (Figure 6, middle panel). DAF-3 has also been reported to negatively regulate daf-7 and daf-8 gene expression in a feedback loop [24]. We find that pdp-1 expression is elevated in daf-3(mgDf90) mutants, suggesting a similar feedback regulation.
PDP-1 Links TGF-β and Insulin/IGF-1 Signaling

Normal Signaling

- DAF-7 activates PDP-1, which phosphatyses DAF-8.
- DAF-14 and DAF-16 are activated.
- DAF-16 nuclear translocation.
- Longevity, Dauer Diapause, and Fat Storage.

Reduced Signaling

- DAF-7 activates PDP-1, which phosphatyses DAF-8.
- DAF-14 and DAF-16 are activated.
- DAF-16 nuclear translocation.
- Longevity, Dauer Diapause, and Fat Storage.

Low Signaling

- DAF-7 activates PDP-1, which phosphatyses DAF-8.
- DAF-14 and DAF-16 are activated.
- DAF-16 nuclear translocation.
- Longevity, Dauer Diapause, and Fat Storage.

Key Proteins:
- DAF-14, DAF-16, DAF-3, DAF-5, DAF-7, PDP-1, PI3K, AKT-1, AKT-2, PPTR-1, PP2A, Insulin(s), TGF-β, DAF-18, P63K, DAF-5.
Figure 6. PDP-1 links TGF-β signaling to the IIS pathway and DAF-16. Top panel: Under favorable environmental conditions, signaling through the TGF-β pathway activates the R-SMAD proteins DAF-8 and DAF-14, which regulate insulin gene expression while antagonizing DAF-3 and DAF-5 function. These insulins may act as agonists and activate IIS, thereby promoting phosphorylation and suppression of DAF-16 activity. In this feed-forward model, the worm undergoes reproductive growth and has a normal life span. Middle panel: PDP-1 negatively regulates TGF-β signaling through dephosphorylation of DAF-8 and DAF-14. Under these conditions, DAF-3 and DAF-5 repress the transcription of agonistic insulins as well as expression of the daf-7 TGF-β ligand and daf-8, leading to further downregulation of the TGF-β pathway. Alternatively, DAF-3 and DAF-5 may promote transcription of potential antagonistic insulins. This results in reduced signaling through the IIS pathway, enhancing DAF-16 nuclear localization. Lower panel: Under low IIS conditions, DAF-16 localization is predominantly nuclear, where it regulates the transcription of hundreds of target genes that act in combination to regulate longevity, stress resistance, dauer formation and fat storage. Under low TGF-β signaling and IIS conditions, DAF-3 and DAF-5 repress these outputs in an opposite manner, with DAF-5 synergizing and DAF-3 antagonizing DAF-16 function (Figure 6 lower panel). With our Q-PCR data, we found that PDP-1 affected only a subset of the DAF-16 target genes tested. These could represent genes that are regulated by DAF-16 and SMAD proteins. SMAD proteins have low affinity for binding DNA, and the orchestration of cellular signals into defined outputs requires their association with additional co-factors [71]. Mammalian SMAD proteins can bind several co-activators and co-repressor proteins to modulate gene transcription [23]. Specifically, a synergy between mammalian FOXO (FOXO1, FOXO3a and FOXO4) and SMAD2/3 was identified for the regulation of several genes involved in cell cycle regulation and the response to stress [72]. Importantly, these interactions required the function of the co-SMAD protein SMAD-4, which is homologous to DAF-3 [72]. Therefore, DAF-3 and DAF-5 could also directly modulate the IIS pathway at the transcriptional level.

A clear interpretation of our results is complicated by three main factors. First, the sheer number of insulins in the worm makes it difficult to assess whether they are functionally distinct. Secondly, the role of temperature in modulating the readouts of the pathway has not been closely explored. For example, we observe the effects of pdp-1 RNAi on daf-2 lifespan at 15°C but the effect decreases at a higher temperature, as the pathway gets more inactive. It is therefore likely that a certain level of signaling through the pathway is required to activate and target PDP-1 to its substrate(s). At higher temperatures such as 20°C or 25°C, there may be extremely low levels of phosphorylated substrate available for PDP-1. Similarly, the effect of a daf-3 null mutation on daf-2 phenotypes is more pronounced at higher temperatures but not at 15°C. Third, the lack of null alleles may provide an incomplete picture of the phenotypes observed. For example, previous studies using non-null alleles of daf-16 only partially suppressed dauer formation of TGF-β pathway mutants and therefore DAF-16 was thought to only affect the IIS pathway [31]. Therefore, temperature, level of signaling and the kind of mutants used (null versus weak) are important additional inputs that need to be considered to better understand the crosstalk between the IIS and the TGF-β pathways.

In conclusion, our studies show that PDP-1 acts through the TGF-β pathway to negatively regulate IIS and promote DAF-16 activity. PDP-1 may mediate this function in part by negatively regulating TGF-β signaling to repress expression of several insulins that feed into the IIS pathway. In humans, dysregulation of TGF-β signaling and the insulin/IGF-1 signaling axis have been implicated in the onset of age-associated diseases such as Type 2 Diabetes and cancer [73–77]. Future studies exploring the interactions between these two pathways as well as the factors that modulate these interactions may ultimately provide a better understanding of the pathophysiology of these diseases.

Materials and Methods

Strains

All strains were maintained at 15°C using standard Ca. elegans techniques [78]. For all RNAi assays, worms were maintained on the RNAi bacteria for two generations except for the assays on the PDHc RNAi. Strains used in this manuscript are listed in Table S4.

RNAi–based assays

RNAi plates were prepared as previously described [39]. All RNAi clones were sequenced and verified before any assays were carried out. L4 worms were picked onto fresh RNAi plates and maintained for two generations prior to the assay, with the exception PDHc RNAi plates. Worms exhibit lethality when maintained on the following RNAi clones: T05H10.6 (E1α), C04C3.3 (E1β), F23B12.5 (E2), or LLC1C3 (E3) [79]. To circumvent this problem, strains were maintained on vector RNAi for two generations and transferred to E1α, E1β, E2 or E3 plates prior to the assay.

Strain construction

For the pdp-1(tm3734);daf-2(e1370) double mutant, daf-2(e1370) males were mated to pdp-1(tm3734) hermaphrodites at 15°C. A total of 30 F1 progeny were picked onto individual plates and allowed to have progeny at 25°C. From the F2 progeny on each plate, dauer worms were selected and transferred to fresh plates and incubated for an additional 24 hours at 25°C. The next day, the dauer worms were allowed to recover at 15°C until they reached adulthood. Subsequently, adult worms were picked onto individual plates and transferred to 25°C and allowed to have progeny. Among the F3 progeny, we observed that some plates had 100% dauer worms at 25°C, while worms in some of the plates exhibited a developmental delay and could not form complete dauers even after 5–6 days at 25°C. Worms from both sets of plates were recovered, picked to individual plates and allowed to self at 15°C. Parents were then tested for pdp-1(tm3734) deletion by PCR. As anticipated, the pdp-1(tm3734);daf-2(e1370) double mutants are unable to form 100% dauers at 25°C.

The daf-2(e1370);pdp-1::gfp strain was made by crossing daf-2(e1370) males to pdp-1::gfp hermaphrodites at 15°C. About 30 F1 animals were transferred to individual plates and allowed to have progeny at 25°C. From the progeny, F2 dauer worms were selected from each plate and allowed to recover at 15°C. The recovered adult worms were then checked for the presence of GFP, and
GFP-positive worms were transferred to individual plates and incubated at 25°C. Plates where 100% of the progeny were dauer and GFP positive were selected and established as the strain for the assays.

Dauer assays
Strains were maintained on RNAi plates for two generations or regular OP50 plates at 15°C. Dauer assays were performed by picking approximately 100 eggs onto 2 fresh plates and incubated at the appropriate temperature. The *pdk-1(sa680), daf-7(e1372) and daf-14(m77)* worms have a strong Egl phenotype. For dauer assays on these strains, gravid adult worms growing on the RNAi plates were washed off the plate with sterile PBS onto a 1.5 mL eppendorf tube. After 2 washes at 2000 g for 30 seconds, the adults were vortexed for 5 mins in 5 mL of 1 N sodium hydroxide and 3% sodiumhypochlorite (final concentration). The samples were then washed twice with sterile PBS and eggs were aspirated with a glass pipette onto fresh RNAi plates. For all dauer assays, plates were scored for the presence of dauer or non-dauers after 3.5–5.5 days, depending upon the strain. Dauer assays were performed at the temperature indicated. Significance was determined by Student’s t-test.

Lifespan assays
Strains were maintained at 15°C and synchronized by picking eggs onto fresh RNAi or OP50 plates. Approximately 60 young adult worms were transferred per plate to a total of three fresh RNAi or regular OP-50 plates containing 5-fluorodeoxyuridine (FUDR) at final concentration of 0.1 mg/mL [80]. All RNAi-RNAi or regular OP-50 plates containing 5-fluorodeoxyuridine (FUDR) at final concentration. Survival was based on lifespan assays were carried out at 15°C similar to the dauer assays. Approximately 50 L4 worms were transferred to fresh RNAi bacteria and the plates were shifted to 20°C for 1 hr. The worms were visualized under a fluorescence microscope (Zeiss AxioScope 2+ microscope). Worms were classified into four categories based on the extent of DAF-16: GFP nuclear-cytoplasmic distribution: completely cytosolic, more cytosolic than nuclear in most tissues, more nuclear than cytosolic in most tissues and completely nuclear.

Psod-3::gfp expression
Quantification of Psod-3::gfp was performed as previously described [39]. *daf-2(e1370); sod-3::gfp* worms were grown at 15°C on RNAi plates as described above. Approximately 30 L4 animals were transferred to fresh RNAi bacteria and shifted to 25°C for 1 hr. The expression of *sod-3::gfp* was visualized using Zeiss AxioScope 2+ microscope. GFP expression was categorized as follows: High: GFP expression present throughout the worm Medium: Weak expression detected in the body of the worm along with the head and the tail Low: Low GFP expression only detected in the head and tail

Transgenic worms
Promoter and ORF entry clones of *pdp-1* obtained from the promoterome and ORFeome were combined using multisite Gateway cloning (Invitrogen) into the pDEST-DD03 or the R4-R2 GFP destination vectors to create the Pdp1-1::gfp or Pdp-1-::pdp-1::gfp::

Quantification of fat staining
For Sudan Black Staining, we used Image J software to measure the average pixel intensity for a 84-pixel radius below the pharynx of each animal in the anterior intestine area. Next, an 84-pixel radius of the background was measured, and subtracted from the values obtained for the staining. At least 10 animals were measured for each RNAi clone. Significance was determined by Student’s t-test.

DAF-16:GFP localization assay
DAF-16 localization assays were performed as previously described [39,52]. *daf-2(e1370); daf-16::gfp* worms were maintained on RNAi plates at 15°C similar to the dauer assays. Approximately 30 L4 worms were transferred to fresh RNAi bacteria and the plates were shifted to 20°C for 1 hr. The expression of *daf-16::gfp* was categorized as follows: High: GFP expression present throughout the worm Medium: Weak expression detected in the body of the worm along with the head and the tail Low: Low GFP expression only detected in the head and tail

RT-PCR experiments
For all RT-PCR experiments, strains were maintained at 15°C. Eggs were obtained from gravid adult worms by hypochlorite treatment described earlier. The eggs were seeded onto large plates maintained at 15°C until the worms entered the L4 stage. The plates were then upshifted to 20°C for 8 hours until they became young adults. Worms were then collected with sterile
1 x PBS and washed twice at 2000 g for 30 seconds. The supernatant was removed, and 0.5 mL of AE buffer (30 mM acetic acid, 10 mM EDTA), 0.1 mL of 10% SDS, and 0.5 mL of phenol was added to the worm pellet and the mixture was vortexed vigorously for 1 min, followed by incubation at 65°C for 4 min. Total RNA was purified by phenol/chloroform extraction and ethanol precipitation. The quality of the RNA isolated was determined by checking the 26 S and 18 S RNA on an agarose gel. 2 μg of total RNA was used for making cDNA using the SuperScript cDNA synthesis kit (Invitrogen, USA). The expression of the DAF-16 target and insulin genes was checked by RT-PCR, using the SYBR Green PCR Master Mix and 7000 Real-Time PCR System (Applied Biosystems, USA). The relative expression of the genes tested was compared to actin as an internal loading control. Significance was determined by Student’s t-test. Primers used for the RT-PCR experiments are listed in Table S5.

**Locomotion assay**

Young adult wild-type and pdp-1(tm3734) worms were picked onto 6 individual plates each. After 5 minutes, the worms were picked off the plate. The average distance covered was calculated by measuring the traces on the bacterial lawn using ImageJ. Significance was determined by Student’s t-test.

**Brood size measurements**

Wild type, daf-2(e1370), pdp-1(tm3734) and pdp-1(tm3734); daf-2(e1370) worms were maintained at 15°C. 5 L4 worms were picked onto individual plates and allowed to lay eggs at 22.5°C. Worms were transferred to a new plate every 12 hours. After 22.5 hours, the parental worms were picked off the plates, and the total number of eggs laid was scored. The number of progeny from these eggs was scored again after 38 hours. The % hatched eggs was calculated as a percentage of the average number of progeny over the average number of eggs laid. Significance was determined by Student’s t-test.

**Software used in this study**

Statistical analyses were performed using JMP and Microsoft Excel. NIH Image J was used for quantification of locomotion and fat storage.

**Supporting Information**

**Figure S1** Verification of RNAi knockdown by Q-PCR. Data shown are from one representative experiment. RNAi knockdown was verified in daf-2(e1370) worms by Q-PCR. For this set, verification of the knockdown for pdkh-2 was performed independently.

**Figure S2** PDP-1 regulates dauer formation independent of the PDHc. Data shown are from one representative experiment. For the dauer assays, Error bars indicate the standard deviation among the different plates within one experiment. A) pdp-1 RNAi significantly suppresses daf-2(e1370) dauer formation (p<0.01), similar to daf-18 RNAi (p<0.01) while E1α RNAi has no effect. pdkh-2 RNAi results in a slight decrease in daf-2(e1370) dauer formation. B) Knockdown of components of the PDHc do not affect daf-2(e1370) dauer formation. RNAi of both, the E1α and E1β or the E2 subunit does not suppress dauer formation like daf-18 RNAi (p<0.01). C) A mutation in pdp-1 suppresses daf-2(e1370) dauer formation, similar to the effect of pdp-1 RNAi. (p<0.03). D) pdp-1 RNAi significantly suppresses daf-2(e1368) dauer formation (p<0.002) similar to daf-18 RNAi (p<0.007). pdkh-2 RNAi has no effect on daf-2(e1368) dauer formation. E) pdp-1 RNAi suppresses dauer formation in daf-2(e1370) mutants (p<0.02) in a RNAi-sensitized background, similar to daf-18 RNAi (p<0.02).

Found at: doi:10.1371/journal.pgen.1001377.s002 (0.96 MB TIF)

**Figure S3** Tissue Expression patterns of PDP-1, A) Expression pattern of pdp-1 as visualized using a Ppd-1::gfp transcriptional fusion strain. DiI staining shows co-localization in amphid neurons. B) The Ppd-1::gfp strain does not show complete overlap with the expression patterns of transcriptional fusion strains of the PDHc, PE1β::gfp and PE2::gfp.

Found at: doi:10.1371/journal.pgen.1001377.s003 (4.41 MB TIF)

**Figure S4** PDP-1 regulates lifespan. Data shown are from one representative experiment. A) pdp-1 RNAi does not significantly reduce the lifespan of wild-type worms (p<0.07). B) pdp-1 RNAi significantly reduces daf-2(e1370) lifespan (p<0.0001) similar to daf-18 RNAi (p<0.0001). C) pdp-1 RNAi significantly reduces age-1(hx546) lifespan (p<0.0001) similar to daf-18 RNAi (p<0.0001). D) Overexpression of pdp-1 increases lifespan (p<0.0001). E) Dosage modulation of pdp-1 can regulate daf-2 lifespan. pdp-1(tm3734); daf-2(e1370) worms live significantly shorter than daf-2(e1370) worms (p<0.0001) while daf-2(e1370); pdp-1::gfp worms live longer (p<0.0001). F) Mutations in daf-14 and daf-7 do not significantly increase lifespan. pdp-1(tm3734) mutants live shorter than wild-type worms (p<0.005).

Found at: doi:10.1371/journal.pgen.1001377.s004 (0.62 MB TIF)

**Figure S5** PDP-1 regulates lifespan in a DAF-16-dependent manner. A) Increased dosage of pdp-1 extends the lifespan of wild-type worms (p<0.005) and this extension is suppressed by daf-16 RNAi (p<0.0001). B) Increased dosage of pdp-1 further extends daf-2(e1370) lifespan (p<0.0001), and this extension is completely suppressed by daf-16 RNAi (p<0.0001).

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**Figure S6** PDP-1 mutants have a slow movement phenotype and reduced brood size. Data shown are from one representative experiment. Error bars indicate the standard deviation among the different plates within one experiment. A) pdp-1(tm3734) mutants have a slow movement phenotype when compared to wild-type worms (p<0.001). This slow movement in the pdp-1(tm3734) mutant can be rescued by expression of a pdp-1::gfp transgene (p<0.002). Lower panel: Traces of wild-type, pdp-1(tm3734), pdp-1::gfp and pdp-1::gfp, pdp-1(tm3734) worms moving on a lawn of OP50. B) Brood size of wild-type, daf-2(e1370), pdp-1(tm3734) and pdp-1(tm3734); daf-2(e1370) animals as scored after 22.5 hours (total number of eggs laid) and 38 hours (total number of progeny). C) The % hatched eggs calculated from the number of progeny and number of eggs laid. pdp-1(tm3734) worms have fewer progeny (p<0.04) when compared to wild-type worms, however, this phenotype is far more severe in pdp-1(tm3734); daf-2(e1370) worms (p<0.005).

Found at: doi:10.1371/journal.pgen.1001377.s006 (0.92 MB TIF)

**Figure S7** PDP-1 regulates stress resistance and fat storage. Data shown are from one representative experiment. Arrows indicate the lower bulb of the pharynx. A) PDP-1 regulates thermotolerance. A mutation in pdp-1 slightly reduces thermotolerance (p<0.06) of wild-type worms but significantly reduces daf-2(e1370) thermotolerance (p<0.05). B) Oil Red O Staining of adult worms. Top panel: Quantification of Oil Red O staining in wild-type and pdp-1::gfp worms. Overexpression of pdp-1 slightly enhances fat storage (p<0.01), and this enhancement is dependent on daf-16 RNAi (p<0.01) but not daf-3 or E1α RNAi. Lower
panel: Oil Red O Staining of young adult worms showing comparable levels of fat between wild-type and pdp-1(tm3734) worms, while pdp-1::gfp young adults show slightly enhanced fat storage. C) Oil Red O Staining of daf-2(e1370) and daf-2(e1370);pdp-1::gfp worms. Top panel: Quantification of Oil Red O staining in daf-2(e1370) and daf-2;pdpa-1::gfp worms. Similar to daf-2(e1370) worms, the fat storage of daf-2(e1370);pdp-1::gfp worms is suppressed by daf-16 RNAi (#p<0.005) but not EliraNAI. daf-3 RNAI slightly reduces the fat of daf-2; pdp-1::gfp but not daf-2(e1370) worms (#p<0.01). Lower panel: Representative images of Oil Red O Staining in daf-2(e1370) and daf-2(e1370);pdpa-1::gfp worms on daf-16, daf-3 and Elir RNAI. D) Quantification of Sudan Black Staining of daf-2(e1370) on different RNAI clones. The increased fat storage of daf-2(e1370) worms is suppressed on daf-18 (#p<0.005), daf-16 (#p<0.005), pdp-1 (#p<0.007) and daf-5 RNAI (#p<0.005).

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Figure S8 PDP-1 positively regulates DAF-16 nuclear localization and activity. A) Quantification of DAF-16 subcellular localization as observed in daf-2(e1370); daf-16::gfp worms on vector, daf-18 and pdp-1 RNAI. B) Quantification of GFP expression in a daf-2(e1370); Pspod-3::gfp reporter strain grown on vector, daf-18, pdp-1, daf-5 and daf-3 RNAI.

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Figure S9 Epistasis analyses using mutants of the IIS pathway. A) pdp-1 RNAI significantly suppresses dauer formation of pdk-1(n6380) mutants (#p<0.01). B) pdp-1 RNAI suppresses dauer formation of daf-2(e1370); akt-1(ok325) double mutants (#p<0.03). C) pdp-1 RNAI suppresses dauer formation of daf-2(e1370); akt-2(ok393) double mutants (#p<0.05).

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Figure S10 Epistasis analyses using mutants of the TGF-β pathway. A) daf-7(e1372) dauer formation is suppressed by pdp-1 RNAI (#p<0.02), daf-18 RNAI (#p<0.005), daf-16 RNAI (#p<0.008) as well as the controls daf-3 RNAI (#p<0.02) and daf-5 RNAI (#p<0.05). B) pdp-1 RNAI has no effect on dauer formation of daf-14(m77) worms (#p<0.1). However, daf-18 RNAI (#p<0.05) and daf-16 RNAI (#p<0.05) result in dauer suppression. C) pdp-1 RNAI has no effect on dauer formation of daf-8(tm825) worms (#p<0.3). Similarly daf-18 RNAI also has no effect on dauer formation (#p<0.1). D) At 25°C daf-16 RNAI can robustly suppress dauer formation of daf-8(tm825) worms (#p<0.009), while daf-3 RNAI only has a partial effect (#p<0.05). E) Dauer formation of daf-2(e1370);daf-3(mgD90) is suppressed by daf-18 RNAI (#p<0.04) but not pdp-1 RNAI (#p<0.2).

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Figure S11 DAF-3 and DAF-5 regulate daf-2(e1370) dauer formation. Data shown are from one representative experiment. Error bars indicate the standard error of the mean within triplicates. A) The increased fat storage of age-1(hx546) worms is suppressed by a mutation in daf-5. Top panel: Oil Red O staining of age-1(hx546) and age-1(hx346);daf-5(e1385) young adult worms. Arrows indicate the lower bulb of the pharynx. Lower panel: Quantification of Oil Red O staining shows significantly reduced fat in age-1(hx546); daf-5(e1385) worms as compared to the age-1(hx546) parental strain (#p<0.009). C) Quantification of Sudan Black staining of daf-7(e1372) L3 worms on different RNAI bacteria. Fat storage of daf-7(e1372) animals is decreased by daf-3 (#p<0.0001), daf-16 (#p<0.001), pdp-1 (#p<0.001), daf-18 (#p<0.0001) and daf-5 (#p<0.0001) RNAI.

Found at: doi:10.1371/journal.pgen.1001377.s012 (0.43 MB TIF)

Figure S12 Crosstalk between the IIS and TGF-β signaling pathways in modulation of lifespan and stress resistance. Data shown for the lifespan assays are from one representative experiment. A) Lifespan of daf-2(e1370); daf-3(mgD90) worms is enhanced over daf-2(e1370) mutants (#p<0.001). pdp-1 RNAI can significantly suppress the lifespan of daf-2(e1370) worms (#p<0.0001) but only has a partial effect on the lifespan of daf-2(e1370); daf-3(mgD90) worms (#p<0.01). daf-18 RNAI significantly reduces lifespan in both strains (#p<0.001). B) age-1(hx346); daf-3(e1385) double mutants live significantly shorter than age-1(hx546) worms (#p<0.0001). Both pdp-1 and daf-18 RNAI significantly reduce the lifespan of both strains (#p<0.0001). C) Survival of adult worms of the IIS and TGF-β pathways after 9.5 hours at 37°C. Data shown is an average of two independent repeats, with error bars indicating the variation between two repeats.

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Figure S14 Q-PCR experiments. Data shown are from one representative experiment. Error bars represent standard error of the mean within triplicates. A) The levels of several insulin genes are elevated in daf-3(mgD90) and pdp-1(tm3734) worms. *p<0.05, **p<0.02, ***p<0.0009. B) The levels of the same set of insulins are markedly decreased in daf-14(m77) worms. *p<0.05, **p<0.0007. C) Insulin levels are significantly decreased in daf-3(mgD90) and pdp-1::gfp strains. *p<0.05, **p<0.01, ***p<0.001. D) Insulin gene regulation is under the control of the IIS pathway. Compared to daf-2(e1370) worms, the levels of several insulins change in pdp-1(tm3734); daf-2(e1370) worms and daf-16(mgD50); daf-2(e1370) worms. *p<0.05, **p<0.0007, ***p<0.0005. E) ins-7 levels are significantly increased in daf-16(mgD50); daf-2(e1370) and pdp-1(tm3734); daf-2(e1370) double mutants, compared to daf-2(e1370). *p<0.04, **p<0.001. F) The levels of several insulin genes are unchanged in daf-16(mgD50) single mutants. *p<0.05.

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Figure S15 DAF-3 regulates pdp-1 expression. A) Q-PCR results showing elevated levels of pdp-1 in daf-3(mgD90) mutants. Also, expression is slightly increased over wild-type worms in daf-2(e1370) mutants but decreased in daf-16(mgD50) worms as well as daf-16(mgD50); daf-2(e1370) worms. Data shown are from one representative experiment. Error bars represent standard error of the mean within triplicates. B) Compared to vector RNAi, GFP expression of the Pdpa-1::gfp transcriptional fusion strain is higher on daf-3 RNAI, and slightly reduced on daf-16 RNAI (10× magnification).

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Table S1 Lifespans of IIS and TGF-β pathway mutants.

Found at: doi:10.1371/journal.pgen.1001377.s016 (0.04 MB DOC)

Table S2 List of insulins tested in this manuscript.

Found at: doi:10.1371/journal.pgen.1001377.s017 (0.04 MB DOC)
Table S3 Summary of trends observed in the Q-PCR Experiments.
Found at: doi:10.1371/journal.pgen.1001377.s018 (0.05 MB DOC)

Table S4 List of strains used in this manuscript.
Found at: doi:10.1371/journal.pgen.1001377.s019 (0.06 MB DOC)

Table S5 List of primers used in this manuscript.
Found at: doi:10.1371/journal.pgen.1001377.s020 (0.13 MB DOC)

References


