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

Sri Devi Narasimhan

University of Massachusetts Medical School

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

Sri Devi Narasimhan¹, Kelvin Yen¹, Ankita Bansal¹, Eun-Soo Kwon¹, Srivatsan Padmanabhan¹, Heidi A. Tissenbaum¹,²*

¹ Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America, ² Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America

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. 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 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 temperature-sensitive 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 PP2A regulatory subunit PPT1 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 (PDP) 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.

PDP-1 Links TGF-β and Insulin/IGF-1 Signaling

In addition to dauer formation, the IIS pathway also regulates longevity, stress resistance and fat storage [17,18]. Mutations indaf-2 andage-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 indaf-18 anddaf-16 [32,34,35,39]. We therefore investigated whether dosage modulation ofpdp-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 bypdp-1 RNAi and slightly reduced by a mutation inpdp-1 [Figure 2A and 2D]. In contrast, the mean and maximal lifespan of long-liveddaf-2(e1370) and age-
PDP-1 Links TGF-β and Insulin/IGF-1 Signaling

Figure 1. PDP-1 regulates daf-2 dauer formation independent of the PDHc. Error bars indicate the standard deviation among the different RNAi plates within one experiment. Data shown are from one representative experiment. (A) pdp-1 RNAi suppresses daf-2(e1370) dauer formation similar to daf-18 RNAi. Dauer formation of daf-2(e1370) is 56.5 ± 8.0% (n = 278) on vector RNAi, 18.9 ± 0.8% (n = 79) on daf-18 RNAi (p < 0.05) and 10.5 ± 5.3% (n = 293) on pdp-1 RNAi (p < 0.05). (B) pdp-1 RNAi suppresses dauer formation of daf-2(e1368) worms similar to daf-18 RNAi. Dauer formation of daf-2(e1368) is 77.1 ± 13.2% dauers (n = 297) on vector RNAi, daf-2(e1368) worms form only 9.4 ± 6.4% (n = 258) dauers on daf-18 RNAi (p < 0.06) and 25.9 ± 3.9% (n = 636) dauers on pdp-1 RNAi (p < 0.05). (C) RNAi of other components of the PDHc including the E1a subunit does not affect daf-2(e1370) dauer formation. Dauer formation of daf-2(e1370) on PDHc RNAi is 23.3 ± 4.1% (n = 282) on vector RNAi, 1.3 ± 0.2% (n = 219) on daf-18 RNAi (p = 0.04), 1.6 ± 0.6% (n = 185) on pdp-1 RNAi (p = 0.03), 13.1 ± 1.0% (n = 233) on pdhk-2 RNAi (p = 0.05), 18.2 ± 2.0% (n = 193) on E1α RNAi, 23.5 ± 0.5% (n = 172) on a combination of E1α and E1β RNAi and 33.3 ± 7.1% (n = 25) on E2 RNAi.

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1(hx546) mutants was significantly reduced by pdp-1 RNAi (Figure 2B and 2C). Similarly, pdp-1(tm3734); daf-2(e1570) 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::gfp; 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); 16::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 ± 0.5 days (n = 93) 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). (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, 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). (C) 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. (D) PDP-1 regulates thermotolerance. Mean survival of wild-type worms is 18.3 ± 0.7 hours (n = 37), pdp-1(tm3734) mutants is 17.1 ± 0.8 hours (n = 27) p < 0.2, 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), p < 0.0007. (F) Oil Red O staining reveals that pdp-1(tm3734); daf-2(e1370) worms store less fat than daf-2 worms across different stages in the worm life cycle: dauer (left), L3 worms (middle) and adults (right). Arrows indicate the lower bulb of the pharynx. (G) Quantification of Oil Red O staining in L3 and young adults of daf-2(e1370), pdp-1(tm3734); daf-2(e1370) and daf-2(e1370); pdp-1::gfp worms. A mutation in pdp-1 significantly reduces daf-2(e1370) fat storage in both, L3s (p < 0.0001) and young adults (p < 0.01). In adult worms, daf-2(e1370); 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 [32] 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.

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). pdp-1(sa680), daf-2(e1370); akt-1(ok525) and daf-2(e1370); akt-2(ok393) mutants were maintained on vector RNAi compared to vector RNAi, similar to daf-16 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 [32] 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 sod-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 sod-3 RNAi, suggesting that pdp-1 does not function at the level of daf-7 (Table 2 and Figure
RNAi had no effect, while vector, pdp-1 indicate a genetic interaction between SMADS S10). Next, we tested dauer formation with mutants of the strain vector RNAi. Interestingly, pdp-1 RNAi had no effect on daf-2(e1370) mutants and again observed that pdp-1 RNAi had no effect, while dfd-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/da8.

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-B pathway is removed due to the daf-3 null mutation, and dauer formation is presumably mediated through activated DAF-16 [39]. Therefore, if pdp-1 was indeed acting in the TGF-B 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 [28]. Therefore, our genetic epistasis analysis supports a model whereby pdp-1 acts in the DAF-7 TGF-B pathway at the level of dfd-8 and daf-14.

TGF-B signaling can modulate the IIS pathway

How does a phosphatase in the TGF-B signaling pathway have such robust effects on the outputs of the IIS pathway and DAF-16?

| Table 1. Genetic epistasis analysis using IIS mutants. |
|------------------------------------------------------|
| **% Dauers ± Std. Dev. (n)**                          |
| Strain | vector RNAI | daf-18 RNAI | pdp-1 RNAI |
|---------|-------------|-------------|-------------|
| pdk-1(m688) | 85.0±4.7 (520) | - | 35.3±2.5 (327)** |
| daf-2(e1370) | 8.3±8.8 (476) | 0 (331) | 5.3±1.0 (241) |
| daf-1(e1370); akt-1(ok395) | 36.9±1.4 (390) | 3.5±0.9 (265)* | 16.0±0.4 (375)* |
| daf-2(e1370) | 75.6±4.8 (247) | 0.3±1.0 (777)** | 17.3±8.2 (597)** |
| daf-2(e1370); akt-2(ok393) | 61.1±15.3 (289) | 4.1±1.7 (308)** | 11.5±3.6 (301)** |

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

*AAs previously reported, pdk-1(m688) mutants survive poorly on daf-18 RNAI.* 

**p<0.01.

*p<0.05.

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A number of studies have previously identified roles for the TGF-B pathway in lifespan and fat storage [7,54,53]. However, genetic epistasis analysis on dauer formation placed DAF-7 TGF-B 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-B 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-B 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-B 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-5(e1376) 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(hs346) worms, with age-1(hs546); 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 storage 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(hs546); daf-5(e1385) had less fat than age-1(hs346) 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 4Ci, 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-B 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-B pathway signaling. For this, we assayed dauer formation of TGF-B 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-

| Table 2. Genetic epistasis analysis using TGF-B signaling mutants. |
|----------------------------------------------------------|
| **% Dauers ± Std. Dev. (n)**                             |
| Strain | vector RNAI | daf-3 RNAI | pdp-1 RNAI |
|---------|-------------|-------------|-------------|
| daf-7(e1372) | 85.3±1.1 (612) | 43.4±0.8 (134)** | 32.2±4.9 (122)** |
| daf-14(m77) | 81.7±5.6 (543) | 18.1±8.9 (441)** | 88.7±1.3 (535) |
| daf-8(m85) | 32.0±9.7 (392) | 2.3±1.8 (396)** | 34.6±9.1 (430) |
| daf-2(e1370); daf-3(mgD90) | 50.8±0.4 (302) | - | 49.5±2.5 (270) |

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

**p<0.01.

*p<0.05.

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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...
Table 3. Dauer formation of TGF-β signaling mutants is regulated by DAF-18 and DAF-16.

| Strain                          | vector RNAi | daf-3 RNAi | daf-18 RNAi | daf-16 RNAi |
|---------------------------------|-------------|------------|-------------|-------------|
| daf-7(e1372)                   | 93.4±3.6 (113) | 52.3±1.0 (683)** | 44.5±1.4 (79)** | 43.1±1.9 (72)** |
| daf-14(m77)                    | 73.1±9.4 (361) | 51.5±4.9 (524)b | 41.5±1.4 (500)* | 23.2±5.0 (152)b |
| daf-8(8155)                    | 99.3±0.5 (441) | 75.7±0.1 (580)** | nt           | 4.3±4.3 (270)* |
| daf-2(e1370); daf-3(mgDf90)    | 47.4±2.0 (364) | -           | 5.9±1.0 (314)** | 0 (240) |

Assays were performed at *22.5°C, **20°C, *25°C and °19.2°C respectively. 
nt – not tested at this temperature. Assays with daf-18 RNAi are in the supplementary data. 
*p<0.01, 
**p<0.005. 
†p<0.05. 
doi:10.1371/journal.pgen.1001377.t003

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 (PDHc). Remarkably, other components of the PDHc in *C. elegans* do not affect daf-2 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 PDHc 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 phosphatases 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, 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-33 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–5C, 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::gfp and pdp-1::gfp animals when compared to wild-type worms. Therefore, dosage modulation of DAF-3 and PDP-1 modulates insulin gene expression. INS-4, for example, has been reported as a positive regulator of DAF-3 and PDP-1 modulates insulin gene expression. INS-4, for example, has been reported as a positive modulation 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.
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 insulin genes, 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
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 function. These insulins may act as agonists and activate IIS, thereby promoting phosphorylation and suppression of DAF-16 activity. In this panel, the worm has a reduced life span. 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 IIS conditions, DAF-3 and DAF-5 play opposite roles. DAF-5 is likely to synergize with DAF-16 and modulate the activity of its target genes. Therefore, the role of DAF-3 in modulating IIS depends upon the level of signaling through the pathway.

Materials and Methods

Strains

All strains were maintained at 15°C using standard C. 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 (El19), C04C3.3 (El19), F23Bl2.5 (E2), or LLC113 (E3) [79]. To circumvent this problem, strains were maintained on vector RNAi for two generations and transferred to El19, El19, 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 eggs were selected and transferred to fresh plates and incubated for an additional 24 hours at 25°C. The next day, the dauers 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% dauers at 25°C, while worms in some of the plates exhibited a lower percentage of dauers. To circumvent this problem, strains were maintained on vector RNAi for two generations and transferred to El19, E2 or E3 plates prior to the assay.

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 insulin genes 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.
GFP-positive worms were transferred to individual plates and incubated at 25°C. Plates where 100% of the progeny were dauers 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(n6860), 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 3 mL of 1 N sodium hydroxide and 3% sodium hypochlorite (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 dauers 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 OP50 plates containing 5-fluorodeoxyuridine (FUDR) at final concentration of 0.1 mg/mL [80]. All RNAi-RNAi or regular OP50 plates containing 5-fluorodeoxyuridine (FUDR) at final concentration of 0.1 mg/mL 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 3 mL of 1 N sodium hydroxide and 3% sodium hypochlorite (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 dauers 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.

**Heat stress assay**

Strains were maintained on RNAi or regular OP50 bacteria at 15°C, as described above. From these plates, approximately 30 young adult worms were picked onto fresh RNAi or regular plates and upshifted to 20°C for 6 hrs. The plates were then transferred to 37°C and heat stress-induced mortality was determined every few hours till all the animals died. Statistical analyses for survival were conducted using the standard chi-squared-based log rank test.

**Fat staining**

Strains maintained RNAi or on regular OP50 plates were synchronized by picking eggs on to fresh plates and grown synchronously at 15°C. The plates were then upshifted to 20°C for 8 hours, at the L2 stage to get L3 worms and at the L4 stage to get young adult worms. Worms were then washed off the plates into microcentrifuge tubes and incubated in 1x PBS buffer for 20 minutes on a shaker at RT. After 2 washes at 3000 rpm for 30 seconds with 1x PBS, the strains were fixed according to the type of staining performed. Oil Red O and Sudan black staining was performed as previously described [39,45,81,82]. After incubation overnight at RT, worms were mounted on slides and visualized using the Zeiss Axioscope 2+ microscope.

**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 animals 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 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 seen 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 promoterome and ORFeome were combined using multisite Gateway cloning. Transgenic worms were generated by ballistic transformation into unc-119(ed3) mutant worms as previously reported (Biorad, USA) [83]. Integrated lines that were obtained were used for further analyses. For the *pdp-1::gfp* translational fusion strain, additional lines were generated by integration of extrachromosomal array lines by UV irradiation as previously described [85]. All translational fusion lines were backcrossed 4× to wild-type prior to analysis.

**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×PBS and washed twice at 2000 g for 30 seconds. The supernatant was removed, and 0.5 mL of AE buffer (50 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 260 and 280 RNA on an agarose gel. 2 ug 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 pdhk-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. pdhk-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(e1370) dauer formation (p<0.002) similar to daf-18 RNAi (p<0.007). pdhk-2 RNAi has no effect on daf-2(e1370) 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).

Figure S3 Tissue Expression patterns of PDP-1. A) Expression pattern of pdp-1 as visualized using a Pdp-1::gfp transcriptional fusion strain. Di-1 staining shows co-localization in amphid neurons. B) The Pdp-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).

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).

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).

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
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(mgDf90) worms is significantly decreased compared to daf-2(e1370) mutants (p<0.001). B) Insulin gene expression is significantly decreased in daf-2(e1370) worms (p<0.001) but only has a partial effect on daf-2(e1370); daf-3(mgDf90) worms (p<0.01). C) Insulin gene expression is significantly decreased in daf-2(e1370); daf-3(mgDf90) worms (p<0.001) but only has a partial effect on daf-2(e1370) mutants (p<0.001). D) Insulin gene expression is significantly decreased in daf-2(e1370) worms (p<0.001) but only has a partial effect on daf-2(e1370); daf-3(mgDf90) worms (p<0.01). E) Insulin gene expression is significantly decreased in daf-2(e1370) worms (p<0.001) but only has a partial effect on daf-2(e1370); daf-3(mgDf90) worms (p<0.01). F) The levels of several insulin genes are elevated in daf-2(e1370) mutants but decreased in daf-2(e1370); daf-3(mgDf90) worms. G) The increased fat storage of daf-2(e1370) worms is suppressed by a mutation in daf-5. H) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. I) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. J) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. K) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. L) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. M) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. N) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. O) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. P) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. Q) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. R) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. S) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. T) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. U) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. V) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. W) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. X) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. Y) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5. Z) The increased fat storage of age-1 worms is suppressed by a mutation in daf-5.

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 RNAi worms on vector, daf-16 and daf-1 RNAi. B) Quantification of GFP expression in a daf-2(e1370); Ppd-3:gfp reporter strain grown on vector, daf-16, daf-1, daf-3 and daf-5 RNAi.

Figure S9 Epistasis analyses using mutants of the IIS pathway. A) pdp-1 RNAi significantly suppresses dauer formation of pdh-1(sa680) mutants (p<0.01). B) pdp-1 RNAi suppresses dauer formation of daf-2(e1370); akt-1(sa325) double mutants (p<0.03). C) pdp-1 RNAi suppresses dauer formation of daf-2(e1370); akt-2(sa293) double mutants (p<0.05).

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-16 RNAi (p<0.005) and daf-5 RNAi (p<0.05) as well as the controls daf-3 RNAi (p<0.02) and daf-5 RNAi (p<0.05). C) pdp-1 RNAi has no effect on dauer formation of daf-14(m77) worms (p<1). However, daf-16 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-2(e1370); daf-3(mgDf90) is suppressed by daf-16 RNAi (p<0.04) but not pdp-1 RNAi (p<0.2).

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 among the different plates within one experiment. A) Dauer formation of daf-2(e1370); daf-3(mgDf376) double mutants is significantly enhanced over daf-2(e1370) worms (p<0.004). B) Dauer formation of daf-2(e1370); daf-3(mgDf90) double mutants is significantly enhanced over daf-2(e1370) worms (p<0.001). C) daf-3 (p<0.03) and daf-5 (p<0.06) mutations enhance and reduce daf-2 dauer formation.

Figure S12 Crosstalk between the IIS and TGF-β signaling pathways in modulation of fat storage. Data shown are from one representative experiment. Error bars indicate the standard error among the different plates within one experiment. A) Oil Red O staining of single and double mutant adult worms of the IIS and TGF-β pathways. Arrows indicate the lower bulb of the pharynx.
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)

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