Published in final edited form as:
Aging Cell. 2008 December ; 7(6): 879–893. doi:10.1111/j.1474-9726.2008.00435.x.

The DAF-2 Insulin-like Signaling Pathway Independently Regulates Aging and Immunity in *C. elegans*

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Summary

The *C. elegans* DAF-2 insulin-like signaling pathway, which regulates lifespan and stress resistance, has also been implicated in resistance to bacterial pathogens. Loss-of-function *daf-2* and *age-1* mutants have increased lifespans (Kenyon *et al.* 1993; Larsen *et al.* 1995; Lithgow *et al.* 1995) and are resistant to a variety of bacterial pathogens (Garsin *et al.* 2003). This raises the possibility that the increased longevity and the pathogen resistance of insulin-like signaling pathway mutants are reflections of the same underlying mechanism (Lithgow 2003; Bolm *et al.* 2004). Here we report that regulation of lifespan and resistance to the bacterial pathogen *Pseudomonas aeruginosa* is mediated by both shared and genetically distinguishable mechanisms. We find that loss of germline proliferation enhances pathogen resistance and this effect requires *daf-16*, similar to the regulation of lifespan. In contrast, the regulation of pathogen resistance and lifespan is decoupled within the DAF-2 pathway. Long-lived mutants of genes downstream of *daf-2*, such as *pdk-1* and *sgk-1* (Paradis *et al.* 1999; Hertweck *et al.* 2004), show wildtype resistance to pathogens. However, mutants of *akt-1* and *akt-2*, which we find to individually have modest effects on lifespan, show enhanced resistance to pathogens. We also demonstrate that pathogen resistance of *daf-2*, *akt-1*, and *akt-2* mutants is associated with restricted bacterial colonization and that *daf-2* mutants are better able to clear an infection after challenge with *P. aeruginosa*. Moreover, we find that pathogen resistance among insulin-like signaling mutants is associated with increased expression of immunity genes during infection. Other processes that affect organismal longevity, including Jun kinase signaling and caloric restriction, do not affect resistance to bacterial pathogens, further establishing that aging and innate immunity are regulated by genetically distinct mechanisms.

Introduction

The genetic regulation of both the rate of aging and resistance to bacterial pathogens are conserved aspects of animal physiology that have been well studied in the nematode *Caenorhabditis elegans* (Kurz & Tan 2004; Ewbank 2006; Antebi 2007). Genes affecting many processes, including neuroendocrine signaling, caloric restriction, and mitochondrial function, have been found to modulate the lifespan of *C. elegans*. The DAF-2 insulin-like signaling pathway regulates lifespan in *C. elegans* (Kenyon *et al.* 1993), and homologous neuroendrocine signaling pathways regulate lifespan in other animals, including mice (Bluher *et al.* 2003; Holzenberger *et al.* 2003; Tatar *et al.* 2003). A number of pathways have been found to regulate innate immunity in *C. elegans*, including p38 MAPK and DAF-2 insulin-like signaling (Ewbank 2006). Loss-of-function mutants of *daf-2* and *age-1* enhance resistance to a variety of bacterial pathogens in *C. elegans* (Garsin *et al.* 2003), and

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insulin-like signaling appears to function in parallel to p38 MAPK in the regulation of innate immunity (Troemel *et al.* 2006).

The insulin-like signaling pathway also regulates dauer formation and resistance to a variety of abiotic stresses in *C. elegans* (Riddle *et al.* 1997). The phenotypes associated with loss-of-function mutations in *daf-2* are suppressed by loss-of-function mutations in *daf-16*, which encodes a FOXO transcription factor. DAF-2 regulates DAF-16 at least in part through the activation of phosphoinositide 3-kinase (PI3-kinase), encoded by *age-1* and *aap-1*. PI3-kinase potentiates the activity of four serine threonine kinases: the PI3K-dependent kinase homolog PDK-1, the Akt/PKB homologs AKT-1 and AKT-2, and the serum- and glucocorticoid-inducible kinase homolog, SGK-1. These kinases appear to have distinct roles in *C. elegans* physiology. Dauer formation is regulated by *akt-1*, *akt-2*, and *pdk-1*, but not *sgk-1*, and oxidative stress resistance is regulated predominately by *sgk-1* and *pdk-1* (Hertweck *et al.* 2004). In contrast, both *pdk-1* and *sgk-1* are individually required for normal lifespan, whereas *akt-1* and *akt-2* appear to function together to regulate lifespan (Hertweck *et al.* 2004; Oh *et al.* 2005; Baumeister *et al.* 2006).

The *C. elegans* germline also regulates lifespan. Worms which lack a germline, either due to mutations which result in loss of germline proliferation or due to laser ablation of the germline, are longer lived (Hsin & Kenyon 1999; Arantes-Oliveira *et al.* 2002). This lifespan extension is DAF-16-dependent, but appears to be regulated in parallel to DAF-2. Some sterile worms are resistant to bacterial pathogens, and this resistance is also DAF-16 dependent (Miyata *et al.* 2008). In contrast to the regulation of lifespan by the germline, which appears to be mediated by signals from the germline stem cells, resistance to bacterial pathogens in these sterile animals appears to be mediated by signals from the embryos.

Extensive analyses using transcriptional profiling have identified target genes whose expression is regulated by DAF-16 and DAF-2 either directly or indirectly (Murphy *et al.* 2003; McElwee *et al.* 2004; McElwee *et al.* 2006). These genes fall into at least two functional groups. The first group includes stress responsive and detoxifying genes, including genes that encode enzymes which neutralize reactive oxygen species (ROS). The second group includes a diverse classes of confirmed and putative antimicrobial effector molecules, including lysozymes (such as *lys-7*), saposin-like genes, such as *spp-1* (Kato *et al.* 2002), glycine/tyrosine-rich antimicrobial peptides, such as *nlp-31* (Couillault *et al.* 2004), ASABF-type antimicrobial peptides, such as *abf-2* (Banyai & Patthy 1998), and thaumatins (such as *thn-2*).

Infection-mediated killing of *C. elegans* by the Gram negative pathogen *Pseudomonas aeruginosa* (hereafter PA14) is associated with the accumulation of bacteria within the intestine (Tan *et al.* 1999), which is accelerated in mutants with enhanced susceptibility to killing, such as the p38 MAPKK mutant *sek-1(km4)* (Kim *et al.* 2002). Aging in *C. elegans* is associated with bacterial accumulation and packing in the intestine. Worms fed heat-killed or UV-killed *E. coli* OP50 have longer lifespans than those fed live OP50 (Gems & Riddle 2000; Garigan *et al.* 2002). Thus, resistance to bacterial infection could enhance lifespan in *C. elegans*. The co-regulation of aging and innate immunity by the DAF-2 insulin-like signaling pathway raises the possibility that the increased longevity and pathogen resistance of insulin-like signaling pathway mutants are reflections of the same underlying mechanism (Lithgow 2003; Bolm *et al.* 2004). Here we present evidence that regulation of lifespan and resistance to PA14 is mediated by both shared and genetically distinguishable mechanisms.

Results

Germline proliferation negatively regulates pathogen resistance through a *daf-16*-dependent mechanism

In fertile animals, *daf-16* is required for the extended lifespan and enhanced pathogen resistance of *daf-2* mutants. *daf-16* mutants, while shorter lived (Lin *et al.* 1997), were reported not to have enhanced susceptibility to *E. faecalis*, *S. aureus*, and *P. aeruginosa* (Garsin *et al.* 2003; Kerry *et al.* 2006; Troemel *et al.* 2006). Some bacterial infections impair egg-laying, resulting in the hatching of eggs within infected animals, a phenotype termed "bagging", which contributes to killing the worm and thereby complicating the interpretation of survival assays (Shapira & Tan 2008). To eliminate bagging, we therefore reassessed the requirement for *daf-16* in immune function using sterile animals. We observed that knockdown of *daf-16* by RNA interference (RNAi) in *rrf-3(pk1426);glp-4(bn2)* animals rendered these animals significantly more susceptible to PA14 (Figure 1A). The *rrf-3(pk1426)* mutation sensitized the animals to RNAi (Simmer *et al.* 2002), and the *glp-4(bn2)* mutation resulted in the development of sterile adults lacking germline proliferation (Beanan & Strome 1992). Similar results were observed with *daf-16* RNAi knockdown in *glp-4(bn2)* worms (Supplemental Table S1). By contrast, *daf-16* RNAi knockdown in sterile animals with intact germline proliferation, *pha-1(e2123)*, resulted in survival kinetics that was indistinguishable from control (Supplemental Table S1). *pha-1(e2123)* animals have intact germline proliferation but are sterile because viable progeny do not develop at non-permissive temperature (Schnabel & Schnabel 1990). Given that *glp-4(bn2)* animals have extended life span (Arantes-Oliveira *et al.* 2002) and the *C. elegans* germline has been shown to negatively regulate adult lifespan in a *daf-16*-dependent manner, such that ablation of the germline increases lifespan only in *daf-16(+)* worms (Hsin & Kenyon 1999; Lin *et al.* 2001), we wondered whether the *C. elegans* germline could also modulate immune function and whether this is dependent on DAF-16. We investigated the effect of loss of germline proliferation on resistance to PA14 by performing *C. elegans* survival assays using worms in which *cdc-25.1* has been knocked down by RNAi (Shapira *et al.* 2006; Shapira & Tan 2008). RNAi knockdown of *cdc-25.1* generates two distinct populations of sterile worms: one with and another without proliferating germlines (hereafter referred to as Emb and Glp, respectively; Supplemental Text). First, we showed that *cdc-25.1* did not affect pathogen resistance beyond that observed in other Glp animals; *glp-4(bn2)* germline-deficient animals treated with *cdc-25.1* dsRNA had similar survival kinetics on PA14 compared to those treated with vector control (Figure 1B). We then compared the survival of Emb and Glp subpopulations of N2 and *daf-16(mu86)* null mutants (Supplemental Table S1) on PA14. The ability of *daf-16(mu86)* and N2 animals to survive infection by PA14 was not significantly different among sterile Emb populations. This corroborates the previous reports (Garsin *et al.* 2003; Troemel *et al.* 2006) that in animals with intact germline proliferation, *daf-16* is dispensable for resistance to bacterial pathogens. In the N2 strain, resistance to PA14 was enhanced by 75% in Glp animals as compared to Emb animals (Figure 1C). However, the enhanced resistance of Glp animals to PA14 was completely suppressed in *daf-16(mu86)* animals, where no significant difference between the survival of Emb and Glp animals could be detected. In contrast, the pathogen sensitive p38 MAPKK mutant *sek-1(km4)* Glp animals were more resistant to PA14 than *sek-1(km4)* Emb animals, indicating that failure to observe a difference between *daf-16(mu86)* Emb and Glp animals is not a result of limited statistical power (Figure 1D). Based on these results, we conclude that germline proliferation negatively regulates resistance to PA14, either directly or indirectly, in a *daf-16*-dependent but *sek-1*-independent manner. This observation is consistent with a previous report that sterile animals, such as *glp-1(e2141)* and *fer-15(b26);fem-1(hc17)*, are resistant to bacterial pathogens in a *daf-16*-dependent manner (Miyata *et al.* 2008). In animals with proliferating germlines, *daf-16* is required for lifespan

regulation but not for resistance to PA14. These observations suggest that despite an overlap in the role of the *daf-2/daf-16* pathway in affecting lifespan and pathogen resistance, some aspects of the regulation could be molecularly distinct.

Lifespan and pathogen resistance are distinctly regulated by components of the insulin-like signaling pathway

To investigate the possible distinct molecular nature of the regulation of lifespan and immunity, we examined additional components of the *daf-2/daf-16* pathway. Previously, using fertile animals, hypomorphic alleles of *daf-2* and *age-1* were reported to be resistant to pathogenic bacteria (Garsin *et al.* 2003). The strength of these mutants varied considerably, with the strongest resistance observed in *daf-2(e1370)* animals. *daf-2(e1370)* mutants however, exhibit other pleiotropic effects, such as reduced brood size and increased incidence of internal hatching (Gems *et al.* 1998; Patel *et al.* 2008) that, as noted earlier, could confound the interpretation of pathogen resistance assays that are based on survival (Shapira & Tan 2008). Using worms rendered Glp by *cdc-25.1* RNAi, we confirmed that *daf-2(e1370)* and *age-1(hx546)* were resistant to PA14 and noted that *daf-2(e1370)* was significantly more resistant to PA14 than *age-1(hx546)* (Figure 2A). Moreover, we found that *daf-2(e1370)* was significantly more resistant to PA14 than other *daf-2* alleles, and pathogen resistance varied more within than between the two phenotypic classes of *daf-2* alleles (Figure 2B). We also observed that *aap-1(ok282)*, a deletion mutant of the p50/p55 PI3K regulatory subunit (Wolkow *et al.* 2002), was resistant to PA14 (Supplemental Table S1). Like *age-1*, *aap-1* loss-of-function mutants are long lived and arrest as dauers when raised at high temperatures (Wolkow *et al.* 2002). Thus, *daf-2*, *age-1*, and *aap-1* are each required for the regulation of both lifespan and resistance to bacterial pathogens.

Downstream of PI3K are four serine threonine kinases: PDK-1, AKT-1, AKT-2, and SGK-1. Loss-of-function mutations in *pdk-1* and *sgk-1* cause lifespan extension (Paradis *et al.* 1999; Hertweck *et al.* 2004). However, there are conflicting reports on the requirement for *akt-1* and *akt-2* in the regulation of lifespan. While the deletion mutants *akt-1(ok525)* and *akt-2(ok393)* are reported not to affect lifespan (Hertweck *et al.* 2004), others have reported that RNAi knockdown of *akt-1* significantly increased mean lifespan (Hamilton *et al.* 2005; Hansen *et al.* 2005). In addition, the *akt-1(ok525);akt-2(ok393)* double mutant is reported to form constitutive dauers at all temperatures, which can be bypassed by *daf-16* RNAi during development, and to have significantly increased lifespan (Oh *et al.* 2005). We wondered if the specificity of these kinases extends to the relationship between pathogen resistance and lifespan. It has been reported that *E. coli* OP50 could grow and proliferate in older worms and contribute to a more rapid death compared to worms grown on dead or nonproliferating bacteria (Garigan *et al.* 2002). This raises the possibility that results obtained from lifespan assays that used live *E. coli* OP50 could be affected by normal lifespan and immune function. We therefore determined the adult lifespans of *akt-1(ok525)* and *akt-2(ok393)* using UV-killed *E. coli* as a food source and monitored survival of these strains on PA14 at 25°C. All assays used worms rendered Glp by *cdc-25.1* RNAi. The *akt-1(ok525)* and *akt-2(ok393)* mutants exhibited a small but significant increase in mean lifespan on UV-killed *E. coli* (Figure 2C and 2D, closed symbols) and a proportionately larger increase in resistance to PA14 (Figure 2C and 2D, open symbols). We speculated that *akt-1* and *akt-2* function partially redundantly for the regulation of pathogen resistance, as has been found for the regulation of dauer formation (Hertweck *et al.* 2004). We were unable to generate *akt-1(ok525);akt-2(ok393)* double mutants, which appeared to be embryonic or early-larval lethal in our experiments. However, we found that double RNAi knockdown of both *akt-1* and *akt-2* enhanced pathogen resistance to a greater extent than either single RNAi (Supplemental Table S1), consistent with a model of partial redundancy for the regulation of pathogen resistance.

Knockdown of *sgk-1* by RNAi increased mean lifespan to more than 160% relative to controls (Hertweck *et al.* 2004). Corroborating this result, we observed that the deletion mutant *sgk-1(ok538)* had a mean lifespan on UV-killed *E. coli* of 230% relative to N2 (Figure 2E, closed symbols). Yet, despite this large lifespan extension, *sgk-1(ok538)* animals were indistinguishable from N2 animals in their ability to survive PA14 infection (Figure 2E, open sybmols). All assays used worms rendered Glp by *cdc-25.1* RNAi. Similarly, we observed that PA14 resistance of animals fed with *sgk-1* dsRNA was indistinguishable from the vector control (Figure 2F). For this assay, *cdc-25.1* RNAi knockdown was achieved by maternal exposure to *cdc-25.1* dsRNA-expressing bacteria followed by egg lay on *sgk-1* RNAi bacteria, thus maximizing the effective concentration of *sgk-1* dsRNA. The long-lived loss-of-function mutant *pdk-1(sa680)* (Paradis *et al.* 1999) also had wildtype-like survival on PA14 in a *cdc-25.1* RNAi Glp background (Figure 2G). *pdk-1(sa680)* carries a missense mutation and thus may possess residual function. To further reduce PDK-1 function, we knocked down *pdk-1* expression using RNAi in a *pdk-1(sa680)* background. Consistent with the suggestion that *pdk-1(sa680)* is a strong loss-of-function mutation (Paradis *et al.* 1999), knockdown of *pdk*-1 did not enhance the pathogen resistance of *pdk-1(sa680)* animals (Figure S3).

To complement our observations with the loss-of-function mutants we tested gain-of-function mutants, *akt-1(mg144)* and *pdk-1(mg142)* for their ability to survive PA14 infection in a *cdc-25.1* RNAi Glp background. *akt-1(mg144)* showed increased susceptibility to PA14, whereas *pdk-1(mg142)* was indistinguishable from wildtype (Figure 2H) despite having a decreased lifespan (Paradis *et al.* 1999). These results indicate that pathogen resistance, like lifespan and dauer formation, is regulated by a distinct subset of the serine threonine kinases downstream of AGE-1 (Figure 2I and 2J). We conclude that both longevity and resistance to bacterial pathogens are regulated by AKT-1 and AKT-2. However, despite their strong effects on lifespan, mutants in *sgk-1* and *pdk-1* do not affect immune function.

Regulation of lifespan and pathogen resistance by DAF-16

The mechanism by which PDK-1, AKT-1, AKT-2 and SGK-1 differentially regulate resistance to pathogen and lifespan is unclear. Genetic and biochemical experiments in worms and mammals indicate that AKT and SGK directly interact with DAF-16 and its mammalian homolog at least in part by the phosphorylation of overlapping but distinct residues on DAF-16 and its mammalian homologs (Kobayashi & Cohen 1999; Brunet *et al.* 2001; Henderson & Johnson 2001). Mutation of these phosphorylation sites results in constitutive nuclear localization of DAF-16, and may be sufficient to cause constitutive dauer formation (Lee *et al.* 2001), but has no detectable effect on lifespan (Lee *et al.* 2001; Lin *et al.* 2001). Using the same transgenic worm strains in which the consensus AKT and SGK phosphorylation sites have been mutated (Lin *et al.* 2001), we likewise did not observe an effect on resistance to PA14 (Supplementary Table S2). Animals without germline have enhanced DAF-16 nuclear localization in their intestine, and this appears to be associated with increased longevity and resistance to pathogen (Lin *et al.* 2001). However, it appears that nuclear localization regulated by AKT and SGK phosphorylation sites is not sufficient to confer increased longevity or enhanced resistance to pathogens.

We also wondered whether lifespan and resistance to bacterial pathogens could be distinctly regulated by the differential activity of DAF-16 in different tissues. DAF-16 has detectable roles in neurons and intestine in affecting lifespan (Wolkow *et al.* 2000; Libina *et al.* 2003; Iser *et al.* 2007). Using the same transgenic worm strains (Libina *et al.* 2003), we examined the role of DAF-16 in regulating pathogen resistance in neurons and intestine. When *daf-16* was expressed as a transgene under its native promoter in *daf-16(mu86); daf-2(e1370)* animals, pathogen resistance was strongly restored (Supplemental Figure S4A,D). In

contrast, when expression of *daf-16* was restricted to either the neurons or the intestine, only partial rescue of the resistance phenotype of *daf-2(e1370)* was observed (Supplemental Figure S4B–D). This pattern of rescue is not substantially different from the pattern that was previously reported for lifespan with these strains (Libina *et al.* 2003). This suggests that a complex interaction between tissues, including neurons and intestine, characterizes the regulation of lifespan and pathogen resistance, but this does not distinguish the regulation of lifespan and pathogen resistance.

Resistance of insulin-like signaling mutants is associated with reduced bacterial colonization, enhanced bacterial clearance, and increased expression of antimicrobial genes

Killing of *C. elegans* by PA14 is associated with the accumulation of bacteria within the intestine (Tan *et al.* 1999), which is accelerated in mutants with enhanced susceptibility to killing, such as *sek-1(km4)* (Kim *et al.* 2002). We hypothesized that pathogen resistant mutants, such as *akt-1, akt-2* and *daf-2* mutants, should accumulate PA14 more slowly than N2. To test this hypothesis, we exposed 1-day-old adult N2, *akt-1(ok525), akt-2(ok393), sgk-1(ok538),* and *daf-2(e1370)* worms to GFP-expressing PA14 for 24 h (Tan *et al.* 1999). The extent of PA14 accumulation was assessed by the presence of GFP fluorescence in the worm intestine. A semi-quantitative scoring scale was used to compare the degree of colonization between populations (Figure 3A). The extent of bacterial colonization in *sgk-1(ok538)* animals was indistinguishable from N2, consistent with wildtype-like pathogen resistance of *sgk-1(ok538)* animals. In contrast, within the *akt-1(ok525)* and *akt-2(ok393)* populations fewer worms were fully or partially colonized and more had undetectable colonization compared to N2. The *daf-2(e1370)* population had further reduced colonization, in accord with its stronger pathogen resistance phenotype in the PA14 survival assays. Differences in feeding and defecation might influence the extent of bacterial colonization. *daf-2(e1370)* animals exhibit reduced pumping (Kenyon *et al.* 1993; Gems *et al.* 1998). Mutations in *eat-2* also result in decreased pharyngeal pumping but no obvious defects in pharyngeal anatomy (Avery 1993). However, *eat-2(ad465)* did not exhibit reduced colonization after 24 h exposure to PA14-GFP and instead exhibited slightly increased bacterial accumulation (Figure S5). This suggests that reduced pharyngeal pumping does not substantially affect bacterial accumulation as measured by PA14-GFP colonization. *daf-2(e1370)* animals also exhibited reduced rates of defecation (Figure S6). Reduced defecation results in increased sensitivity to PA14 (Shapira & Tan 2008), presumptively due to a reduced ability to clear pathogenic bacteria. Thus, reduced defecation in *daf-2(e1370)* cannot explain the reduced colonization of *daf-2(e1370)* mutants.

We further assessed the immune competence of *daf-2* pathway mutants by determining their ability to clear an infection. For the clearance assay, animals were initially exposed to PA14-GFP for 24 h and then shifted to an *E. coli* food source for 24 h to recover. The extent of colonization was assayed before and after the shift in two categories: detectable and undetectable colonization. The ratio of colonization before and after shift was used as a metric of bacterial clearance (Figure 3B). For each strain tested, colonization was significantly reduced after the 24 h shift to *E. coli*. However, the magnitude of the drop in colonization was significantly larger for *akt-1(ok525)* and *akt-2(ok393)* than N2 (log-linear analysis, $p < 0.05$) whereas N2 and *sgk-1(ok538)* were not significantly different in their ability to clear PA14-GFP. The extent of PA14-GFP colonization in *daf-2(e1370)* was too low to measure accurately in the PA14-GFP clearance assay. To overcome this problem, the magnitude of the effect of *daf-2(e1370)* on the accumulation of PA14 was further quantified by enumerating live intestinal PA14 as colony forming units (CFUs). Following 24 h exposure of N2 and *daf-2(e1370)* to PA14, worms were disrupted and CFUs of PA14 were quantified from the worm lysates. *daf-2* worms had significantly lower CFUs than N2

animals (Figure 3C), confirming that they were colonized to a lesser extent. For the clearance assay, *daf-2* and N2 animals were initially exposed to PA14 for a predetermined period and then shifted to an *E. coli* food source. The number of live bacteria within each worm strain was determined immediately before and 24 h after the shift to *E. coli*. Because equal exposure time resulted in unequal initial colonization between *daf-2* and N2 animals (Figure 3C), we exposed *daf-2* and N2 worms to PA14 for 24 h and 18 h, respectively. Despite higher PA14 inoculums in *daf-2* than N2 animals (Figure 3D, initial), *daf-2* worms were able to reduce intestinal PA14 by more than 1000 fold following the shift to *E. coli* (Figure 3D, shift). In contrast, the PA14 load in N2 did not change significantly 24 h after shift to *E. coli* (Figure 3D, shift). In animals that were shifted to PA14 for 24 h, PA14 load did not decrease in either strain (data not shown), indicating that the decrease in live PA14 in *daf-2(e1370)* animals is a function of its increased ability to clear the pathogen from the intestine. Thus, the enhanced resistance of *daf-2(e1370)* worms to PA14 is associated with a corresponding enhanced ability to restrict bacterial colonization and to clear pathogens.

A microarray analysis of insulin-like pathway mutants identified putative antimicrobial genes as potential DAF-16 transcriptional targets (Murphy *et al.* 2003). Thus, a plausible mechanism for the enhanced ability of *daf-2* animals to restrict bacterial colonization is the constitutively elevated expression of proteins with antibacterial functions. We have observed that several genes that are up-regulated in *daf-2* mutants, including *thn-2*, *spp-1*, and *lys-7*, are necessary to restrict colonization by PA14 (T. Kawli and M.-W. Tan, unpublished data). To demonstrate that resistance of *daf-2* pathway mutants is correlated with the expression of antimicrobials, we compared the expression levels of five candidate antimicrobial genes in N2 and *daf-2*-pathway worms that were exposed to OP50 or PA14 for 12 h using quantitative RT-PCR. The criteria for selecting these genes are as follows. First, they are differentially regulated in *daf-2* mutants ((Murphy *et al.* 2003) and E. Evans, S. Slutz and M.-W. Tan, unpublished data) or respond to PA14 infection (Shapira *et al.* 2006; Troemel *et al.* 2006). Second, they either have demonstrated antimicrobial activity (the defensin-like gene *abf-2* (Banyai & Patthy 1998), the saposin gene *spp-1* (Kato *et al.* 2002), and the neuropeptide-like protein *nlp-31* (Couillault *et al.* 2004)) or are homologous to proteins shown to have antimicrobial activity and when knocked down resulted in increased sensitivity to PA14 without affecting normal lifespan (*thn-2* (O'Rourke *et al.* 2006; Shapira *et al.* 2006) *lys-7* (T. Kawli and M.-W Tan, unpublished)). *spp-1* and *abf-2* expressions were also induced during *Salmonella typhimurium* infection and each is required to prevent colonization by this enteric pathogen (Alegado & Tan 2008). Following 12 h exposure to OP50, antimicrobial gene expression was elevated in *akt-1(ok525)*, *akt-2(ok393)* and *daf-2(e1370)* but not *sgk-1(ok538)* (Figure 3E). Expression of *abf-2* was significantly higher in *akt-2(ok393)* and *daf-2(e1370)*, *spp-1* was expressed at higher levels in *akt-2(ok393)* and *thn-2* was expressed at higher levels in *akt-1(ok525)*. To confirm that antimicrobial gene expression remained elevated during PA14 infection, we examined gene expression following a 12 h exposure to PA14. The expression levels of *abf-2*, *spp-1*, *nlp-31*, *thn-2* and *lys-7* were each significantly higher in *daf-2(e1370)* worms than in N2 worms (Figure 3F). The average expression of each of the five candidate immunity genes was also higher in both *akt-1(ok525)* and *akt-2(ok393)* compared to N2 (Figure 3F), consistent with their pathogen resistance phenotypes. In contrast, the expression of these immunity genes in *sgk-1(ok538)* was mixed between up and down-regulation. While *spp-1* was expressed at significantly higher levels, *abf-2* and *lys-7* expression were significantly lower in *sgk-1(ok538)*, and the overall pattern of expression of antimicrobial genes in *sgk-1(ok538)* was not significantly different than N2 (Figure 3F), consistent with the wildtype-like pathogen resistance of *sgk-1(ok538)* mutants. These findings indicate that the expression of these immunity genes as an aggregate during infection is predictive of bacterial colonization and survival, and may underlie the differences between AKT and SGK in immune modulation.

Lifespan extension is uncoupled from pathogen resistance in known aging mutants

To further dissociate the genetic basis for lifespan and immune regulation, we tested additional well-characterized aging mutants for resistance to PA14. Mutations in *clk-1*, a gene necessary for ubiquinone synthesis, alter developmental and physiological timing and extend lifespan in *C. elegans* (Lakowski & Hekimi 1996). Survival of *clk-1(e2519)* on PA14 was not significantly different than N2 in a *cdc-25.1* RNAi Glp background. (Figure 4A). Mutations in *eat-2* affect the rate of pharyngeal pumping, and *eat-2* is widely used as a genetic model for lifespan extension due to caloric restriction (Lakowski & Hekimi 1998). As with *clk-1*, we did not observe increased resistance to PA14 in *eat-2(ad465)* animals in a *cdc-25.1* RNAi Glp background (Figure 4B). This finding is not unexpected because we previously found that an *eat-1* mutant, which also exhibits a reduced rate of pharyngeal pumping (Avery 1993) and increased lifespan (Lakowski & Hekimi 1998), was indistinguishable from wildtype for resistance to PA14 (Tan *et al.* 1999). Both *clk-1* and *eat-2* are reported to function independently of *daf-2* insulin-like signaling to increase lifespan (Lakowski & Hekimi 1996; Lakowski & Hekimi 1998; Houthoofd *et al.* 2003). Consistent with this observation, neither gene appears to be required for the regulation of resistance to bacterial pathogens.

The c-Jun N-terminal kinase pathway positively regulates lifespan in parallel to the DAF-2/AKT-1/AKT-2 pathway but both converge on DAF-16 (Oh *et al.* 2005). Loss-of-function *jnk-1(gk7)* and *jkk-1(km2)* mutants have shortened lifespans (Oh *et al.* 2005). However, unlike *daf-16(mu86)*, neither mutant was more sensitive than N2 as *cdc-25.1* RNAi Glp animals to PA14 (Figure 4C–D). Complementing these results, the absence of a pathogen sensitivity phenotype for a *jnk-1* mutant with intact germline was previously reported (Kim *et al.* 2002). Our analysis with Glp animals further shows that neither *jnk-1* nor *jkk-1* is required for germline-regulated pathogen defense.

Discussion

We showed that resistance to bacterial pathogens and lifespan can be uncoupled within the *daf-2* pathway (Figure 1–Figure 3) and in a variety of aging mutants (Figure 4). Pathogen resistance and lifespan are concordantly regulated by the DAF-2 insulin-like receptor and by AGE-1/AAP-1 PI3 kinase signaling (Figure 2A and Supplemental Table S1). Downstream of DAF-2 and AGE-1, four serine theonine kinases affect the activity and localization of the forkhead transcription factor DAF-16, which directly or indirectly regulates the expression of genes involved in the regulation of life span and immunity. We showed that these serine threonine kinases differentially regulate pathogen resistance and lifespan (Figure 5). These observations support two direct conclusions regarding the regulation of lifespan and pathogen resistance. First, by showing that deletion mutations in *akt-1* or *akt-2* have increased lifespan on UV-killed *E. coli*, an assay that eliminated the possible contribution of immune function for survival on live *E. coli*, we can conclude that AKT-1 and AKT-2 regulate lifespan at least in part independently of their regulation of pathogen resistance. Second, we conclude that SGK-1 and PDK-1 regulate lifespan independently of pathogen resistance because loss-of-function mutations in *sgk-1* and *pdk-1* enhanced lifespan without affecting pathogen resistance. Taken together, these findings indicate that lifespan and pathogen resistance are regulated by genetically distinguishable mechanisms.

The interpretation of the wildtype-like pathogen resistance of the *pdk-1(sa680)* mutant depends on whether *pdk-1(sa680)* retain retains residual PDK-1 activity. Biochemical evidence indicates that PDK-1 functions upstream of AKT-1, AKT-2, and SGK-1. However, the developmental requirement for *pdk-1* can be bypassed by overexpression of *akt-1* or *akt-2* but not *sgk-1* in a *pdk-1(sa680)* background (Hertweck *et al.* 2004). We observed that RNAi knockdown of *pdk-1* in a *pdk-1(sa680)* background does not enhance pathogen

resistance, consistent with *pdk-1(sa680)* being a strong loss-of-function or null mutant. If *pdk-1(sa680)* is a null allele, then the pathogen resistance phenotypes of *akt-1*, *akt-2*, *sgk-1*, and *pdk-1* loss-of-function mutants (Figure 2) suggest that AKT-1 and AKT-2 can act independently of PDK-1 in the regulation of pathogen resistance. Alternatively, *pdk-1(sa680)* may retain residual signaling to AKT-1 and AKT-2 but not SGK-1. Resolution of this issue requires the identification of a *pdk-1* null allele. These observations illustrate that pathogen resistance provides an alternative phenotype with which to delineate insulin-like signaling.

DAF-2 and DAF-16 regulate worm physiology as part of an intricate signaling pathway with contributions from tissue-specific and yet-unidentified components. We failed to find evidence that regulation of pathogen resistance and lifespan are distinguished by the AKT/SGK phosphorylation sites on DAF-16 or by the tissue specific function of DAF-16 in insulin-like signaling. However, we could not rule out the possibility that one or both of these mechanisms contributes to the differential regulation of pathogen resistance and lifespan. An alternative hypothesis which has been examined elsewhere (Paradis & Ruvkun 1998; Lin *et al.* 2001; Hertweck *et al.* 2004) is that AKT-1/AKT-2/SGK-1 competes with an alternative parallel branch of the DAF-2 pathway. Activation of a parallel pathway that converges on DAF-16 could account for the superlative pathogen resistance phenotypes of *daf-2(e1370)* mutants we observed.

We have also found evidence of a role for DAF-16 in pathogen resistance as a target of signaling regulated by germline proliferation in addition to its role downstream DAF-2. Our results indicate that *daf-16* is necessary for the enhancement of resistance in germline-deficient animals, which parallels the observation that lifespan is regulated by germline signaling (Hsin & Kenyon 1999; Lin *et al.* 2001). In animals with proliferating germlines, however, *daf-16* is required for lifespan regulation but not for resistance to PA14. In contrast, *sek-1*, *jnk-1*, and *jkk-1* do not appear to suppress the pathogen resistance of Glp animals. The observation that a *daf-16* null mutant completely suppresses the pathogen resistance associated with loss of germline proliferation suggests that the effect of eliminating germline proliferation on pathogen resistance is mediated by activation of DAF-16.

Recently, analyses of several mutants identified from an enhanced pathogen resistance (Epr) screen suggest that sterility contributes to pathogen resistance in a DAF-16-dependent manner (Miyata *et al.* 2008). In contrast to the regulation of lifespan by the germline, which appears to be mediated by signals from the germline stem cells, the authors suggest that resistance to PA14 in these sterile animals is mediated by signals from the embryos. It will be interesting to determine what distinguishes sterile strains in which PA14 susceptibility is *daf-16*-dependent, such as *glp-1(e2141)* and *fer-15(b26);fem-1(hc17)*, from those strains in which PA14 susceptibility is *daf-16* independent, such as *pha-1(e2123)* and *cdc-25.1* Emb (Figure 1 and Table S1). One possible explanation is that *pha-1(e2123)* and *cdc-25.1* Emb animals possess fertilized embryos, whereas fertilization does not occur in any of the sterile animals in which *daf-16*-dependent pathogen resistance is observed. If the embryo-signal hypothesis of sterile pathogen resistance is confirmed, it would represent another instance of the distinguishable regulation of pathogen resistance and lifespan. Like *daf-2* insulin-like signaling, the pathogen resistance of sterile mutants represents the regulation of pathogen resistance and lifespan by convergent signals acting on DAF-16.

We examined two mechanisms that are associated with pathogen resistance in insulin-like signaling mutants. We have shown that the pathogen resistance of insulin-like signaling mutants is associated with reduced bacterial colonization and enhanced expression of antimicrobial genes. The enhanced resistance of *daf-2* pathway worms to PA14 (Figure 2) is

associated with a corresponding enhanced ability to restrict bacterial colonization (Figure 3A,C) and to clear pathogens (Figure 3B,D). The expression of putative antimicrobial genes during infection is predictive of bacterial colonization and survival and may underlie the differences between *akt-1*, *akt-2*, and *sgk-1* in immune modulation (Figure 3E,F). Our data suggest a model wherein pathogen resistance of *daf-2* pathway mutants is caused in part by reduced bacterial colonization due to enhanced expression of immunity genes.

An ROS resistance model has also been proposed to explain the pathogen resistance of *daf-2* mutants, such that resistance to oxidative stress is partially required for resistance to pathogen (Chavez *et al.* 2007). It has been reported that *sgk-1* and *pdk-1* mutants have enhanced ROS resistance, but *akt-1* and *akt-2* mutants do not, which may account for the effects of these mutants on lifespan (Hertweck *et al.* 2004). We speculate that enhanced ROS resistance alone is insufficient for enhanced resistance to *P. aeruginosa* and that enhanced expression of antimicrobials may be necessary. One possibility is that the superlative resistance of *daf-2(e1370)* is mediated by enhancements of both ROS resistance and antimicrobials. Thus, the regulation of both ROS resistance and antimicrobial gene expression by the *daf-2* pathway may explain the convergence of pathogen resistance and lifespan regulation.

Our results indicate that merely being long lived is not sufficient for resistance to bacterial pathogens, nor are short-lived mutants necessarily sensitive to pathogens (Figure 4). In addition, several examples of pathogen-sensitive or pathogen-resistant mutants with normal lifespans have been reported. Loss of the GATA transcription factor *elt-2* in adults enhances sensitivity to pathogen without shortening lifespan (Shapira *et al.* 2006), and a relatively normal lifespan is reported for the pathogen-sensitive *sek-1(km4)* and *pmk-1(km25)* mutants (Kim *et al.* 2002;Troemel *et al.* 2006). Also, the necrosis-deficient mutants *vha-12(n2915)* and *unc-32(e189)* are resistant to two Gram negative bacteria but have normal lifespan (Wong *et al.* 2007).

The *C. elegans* model of aging and innate immunity may provide insights into the regulation of these processes in other organisms, including humans. The human gut is colonized by hundreds of distinguishable bacterial strains (Gill *et al.* 2006), and the composition of this intestinal microbiota varies with genotype, age, diet, and health (Lupp & Finlay 2005; Turnbaugh *et al.* 2006). Moreover, decline in immune function is an important facet of human aging (Ginaldi *et al.* 2001) and pathogenesis is a major cause of mortality in older adults (Heron 2007). In *C. elegans*, aging is also associated with a decline in pathogen resistance (Kurz & Tan 2004), and post-reproductive worms are compromised in their ability to respond transcriptionally to PA14 exposure (E. A. Evans and M.-W. Tan, unpublished observation).

Aspects of the molecular regulation of aging and innate immunity are also conserved from worms to human. Insulin-like signaling is an evolutionarily conserved regulator of lifespan, and reduction of insulin-like signaling increases lifespan in flies (Clancy *et al.* 2001; Clancy *et al.* 2002) and mice (Bluher *et al.* 2003; Holzenberger *et al.* 2003; Tatar *et al.* 2003). Reduced insulin-like signaling also increases resistance to bacterial pathogens in *Drosophila* (Libert *et al.* 2008). However, in contrast to *C. elegans*, canonical antimicrobial peptides do not appear to be expressed at higher levels in *Drosophila* insulin-like signaling mutants (Libert *et al.* 2008). Also, Akt and FOXO, the fly homologues of AKT-1/2 and DAF-16, play a causal role in a bacterial infection-induced wasting (Dionne *et al.* 2006). In mammals, insulin and IGF-1 signaling have broad effects on the innate and adaptive immune systems (Heemskerk *et al.* 1999; Kelley *et al.* 2007). Dietary restriction is another conserved regulator of lifespan. We observed no enhancement of pathogen resistance in worms subjected to a genetic form of dietary restriction (Figure 4B). Similarly, dietary restriction

does not enhance pathogen resistance in *Drosophila* (Libert *et al.* 2008). In mammals, including humans, reports of the effects of dietary restriction on immunity are mixed (Jolly 2007) but suggest either no effect (McFarlin *et al.* 2006), a marginally positive effect (Rankin *et al.* 2006), or negative effects (Gardner 2005) on immune function. Because many aspects of the molecular regulation of aging and innate immunity are conserved from worms to mammals, it will be interesting to determine if our finding that the genetic regulation of lifespan and pathogen resistance in *C. elegans* are distinguishable may apply to other organisms as well.

Experimental Procedures

Strains

Caenorhabditis elegans strains were obtained from the *Caenorhabditis* Genetics Center (CGC) unless otherwise mentioned. *sgk-1(ok538), akt-1(ok525)* and *akt-2(ok393)* were backcrossed to N2 4x and an independent backcrossed line of each was gratefully received from M. Hertweck and R. Baumeister of the University of Freiburg (Hertweck *et al.* 2004). Within experiments, worm strains were grown at matched temperatures. *daf-2(e1370)* were grown at 20°C. *rrf-3(pk1426);glp-4(bn2), glp-4(bn2)*, and *pha-1(e2123)* were grown at 15°C and shifted to 25°C by L4. *age-1(hx546)* and *pdk-1(sa680)* were grown at 15°C until adulthood. *sgk-1(ok538), akt-1(ok525), akt-2(ok393), clk-1(e2519)* and other strains were grown at 25°C unless matched with strains grown at lower temperatures.

Pseudomonas aeruginosa strains included the clinical isolate PA14 and PA14-GFP, an isogenic GFP-expressing strain (Tan *et al.* 1999). *Escherichia coli* was a streptomycin-resistant derivative of OP50, OP50-1, obtained from the CGC. Bacteria expressing dsRNA directed against *daf-16*, *akt-1*, and *cdc-25.1* were part of a *C. elegans* RNAi library expressed in *E. coli* (Geneservice, Cambridge, U.K.). Bacteria expressing dsRNA directed against *akt-2* and *sgk-1* were part of a *C. elegans* RNAi library expressed in *E. coli* (Open Biosystems, Huntsville, Alabama). Bacterial expressing dsRNA directed against *pdk-1* was a gift from W. Iser and C. Wolkow (Gami *et al.* 2006). All bacterial strains were cultured by shaking at 37°C.

cdc-25.1 RNAi knockdown

RNAi knockdown of *cdc-25.1* in egg-laying adults was used to produce a mixed population of animals with or without a proliferating germline (Glp), which could be clearly distinguished at the 1-day old adult stage using a stereomicroscope. Those animals with a germline are fertile, but the eggs they lay will not hatch (Emb). The distinction between Glp and Emb animals are confirmed by DIC microscopy. Glp animals are readily confirmed by the absence of the gonads and germline and by the absence of eggs laid on plates containing only Glp animals. Emb animals are recognizable as young adults by the presence of embryos in the uterus of Emb worms. Within the population of *cdc-25.*1(RNAi) animals, a small percentage of animals have ambiguous classification as Glp or Emb, and these animals are not used in any of the experiments we performed. Two sequential 4 h egg lays on RNAi plates at 25°C were used as described (Shapira *et al.* 2006). Unless noted, Glp animals were selected for the *C. elegans* survival assays.

C. elegans survival assays

Assays to determine the ability of *C. elegans* to survive PA14 infection were performed as described (Shapira & Tan 2008). Briefly, PA14 was grown overnight in King's Broth containing 100 mg/ml rifampicin at 37°C. 10 μl was spread on modified nematode growth (NG) media and grown for 24 h at 37°C. Worms were infected at 25°C by feeding on PA14 lawns. Kaplan-Meier survival analysis was performed using StatView 5.0.1. The Mantel-

Cox logrank test was used to assess statistical significance of differences in survival. Only p-values < 0.01 were considered significant to minimize type I errors. Mean time to death and standard error of the mean was calculated in StatView and then normalized to N2 for graphical comparison (Table S1–2).

For lifespan assays, UV-killed bacteria was prepared as described (Gems & Riddle 2000). Briefly, OP50-1 was grown in LB with 100 mg/ml streptomycin, concentrated 20X, and 80µl was spread onto NG plates. Plates were placed at ~23°C for 16h, then exposed to UV in a Stratagene Stratalinker 2400 for 4 min.

Bacterial accumulation assay

Young adult worms were exposed to PA14-GFP for 24h at 25°C, and then examined at 40X magnification for intestinal accumulation of GFP using a Leica MZFLIII stereomicroscope. Individual worms were categorized as fully colonized, partially colonized, or undetectable colonization. These categories have been validated against colony-forming unit counts (CFUs). Specifically, 6 worms from each category were individually enumerated for CFUs; with fully colonized, partially colonized, or undetectable colonization corresponding to averages of 4.22×10^4, 2.45×10^4 and 3.48×10^3 colony forming units of PA14-GFP, respectively (data not shown). Population proportions of these categories were compared by Chi-square test using Statview 5.0.1. Experimental replicates were pooled for graphical display and a summary statistical analysis.

Colony forming unit measurement

Pools of ten worms were picked into M9 containing 25 mM levamisole, washed twice in M9 containing 25 mM levamisole, then exposed to 25 mM levamisole, 1mg/ml ampicillin, and 1mg/ml gentamycin in M9 for 1 h to kill external bacteria. Worms were washed again in M9 containing 25 mM levamisole and disrupted in M9 containing 1% Triton-X100 using a motorized pestle. Serial dilutions of worm lysate were grown overnight on LB containing 100µg/ml Rifampicin at 37°C and colony forming units were quantified. At least three replicates for each condition were processed in parallel. For shift experiments, worms were moved to OP50-1 lawns on NG agar containing 300µg/ml streptomycin.

RNA extraction and qRT-PCR

Worms were grown until young adults at 20°C on OP50-1, then moved to 6–8 modified NG plates containing 10 µl PA14 grown overnight. After a 12 h exposure at 25°C, worms were resuspended in M9, washed 3 times, resuspended in 1 ml of Trizol, and stored at −80C until use. Total RNA was extracted as described (Reinke *et al.* 2000) and DNase treated using TurboDNAse (Ambion, Austin TX). RT-PCR was performed with the Bio-Rad iScript One-Step RT-PCR Kit with SYBR Green in 25 µl reactions using a Bio-Rad iCycler according to the manufacturer's instructions (BioRad Laboratories, Hercules, CA). The average cycle threshold of three primer pairs (*ama-1*, F44B9.5, and pan actin (*act-1*,*3*,*4*)) was used as a loading control correction. Summary statistics and statistical tests were calculated from N2-normalized cycle threshold values prior to conversion to relative fold change. Calculations were performed with Microsoft Excel 2003.

Primers

Whenever possible, primers were designed to amplify a sequence found in spliced cDNA but not genomic DNA by having one of the primer pairs overlap an exon junction. Primer design was aided with the program AutoPrime (Wrobel *et al.* 2004). Primer sequences are available from the authors upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank A. Brunet, A. Fire, and the members of the Tan lab, for critical comments, discussions and technical advice; S. Slutz for assistance with RT-PCR; T. Kawli for assistance with microscopy; B. Hamlin for assistance with survival assays; the Caenorhabditis Genetics Center, the *C. elegans* Gene Knockout Consortium, M. Hertweck, and R. Baumeister for worm strains; W. Iser and C. Walkow for RNAi clones. This work was supported by grants from the National Institutes of Health (M.-W.T.). E.A.E. was supported by a Stanford Graduate Fellowship and a National Science Foundation Graduate Research Fellowship. W.C.C. was supported by a Stanford Graduate Fellowship and a Howard Hughes Medical Institute Graduate Fellowship.

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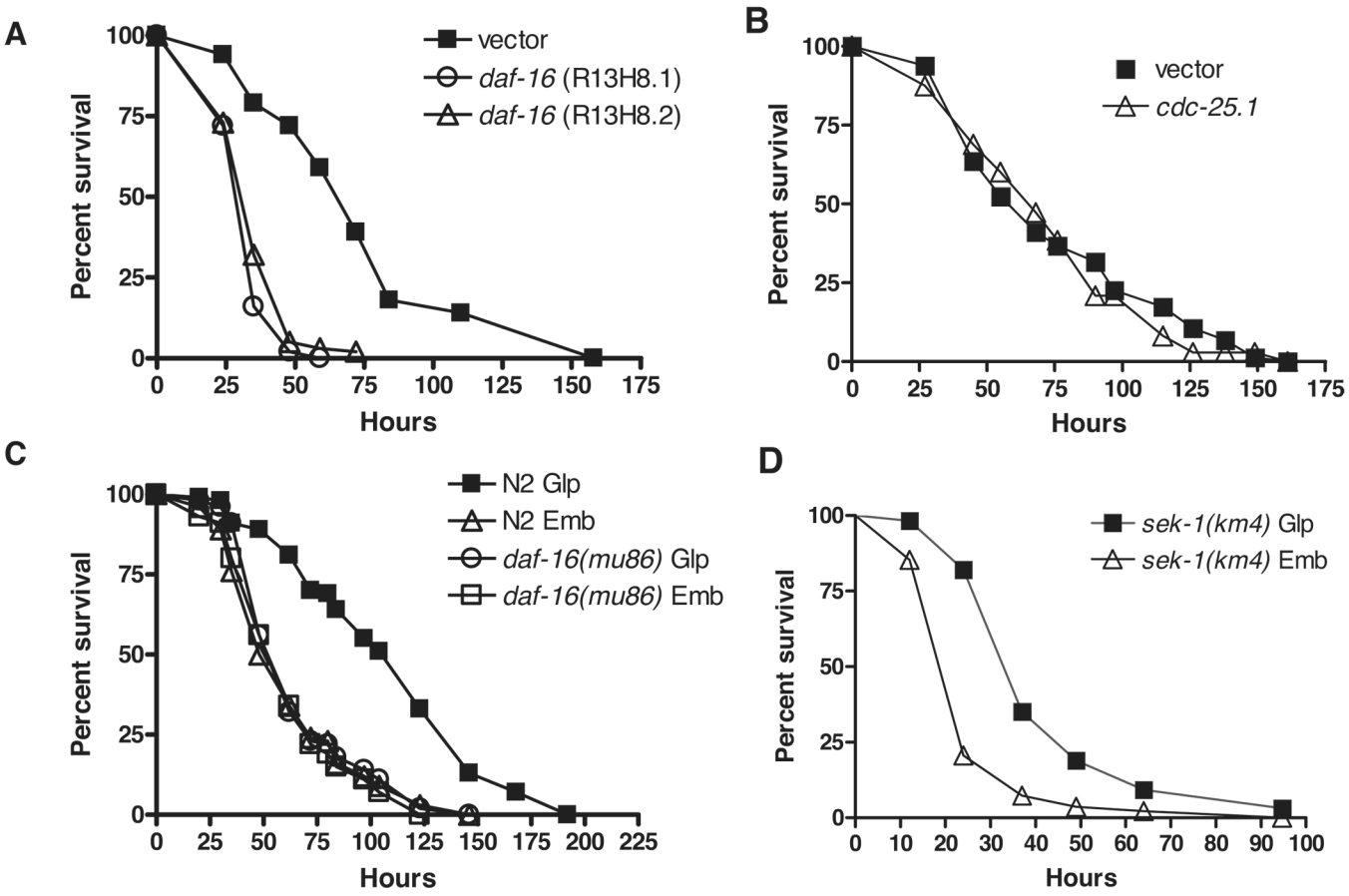

Figure 1. Germline proliferation regulates pathogen resistance through a *daf-16*-dependent mechanism

Survival of worms on PA14 was monitored over time at 25°C. (A) RNAi knockdown of *daf-16* causes enhanced susceptibility to PA14 in worms without a proliferating germline. Enhanced susceptibility of *rrf-3(pk1426);glp-4(bn2)* worms to pathogen was observed with two independent RNAi constructs compared to vector control (logrank test, $p < 0.0001$): R13H8.1, which targets all isoforms of *daf-16*, and R13H8.2, which targets the most broadly-expressed isoforms (Supplemental Figure S1). Survival of worms treated with R13H8.1 and R13H8.2 were indistinguishable (logrank, $p = 0.04$). Similar results are observed with *daf-16* RNAi knockdown in *glp-4(bn2)* worms (Supplemental Table S1). (B) RNAi knockdown of *cdc-25.1* does not affect the ability of *glp-4(bn2)* worms to survive infection by PA14 (logrank, $p = 0.26$, compared to vector control). (C) Enhanced resistance to PA14 due to loss of germline proliferation is *daf-16*-dependent, but *daf-16* is dispensable for resistance to pathogen in animals with intact germline proliferation. In N2 populations, Glp worms were more resistant to PA14 infection than Emb worms (logrank test $p < 0.0001$), but in *daf-16(mu86)* populations Emb and Glp were not significantly different (logrank test $p = 0.08$). In comparison to N2 Emb, *daf-16* Emb was not significantly different (logrank test $p = 0.07$). (D) The enhanced pathogen resistance due to loss of germline proliferation does not require *sek-1*. Compared to *sek-1(km4)* Emb, the mean survival of *sek-1(km4)* Glp is significantly enhanced (logrank, $p < 0.0001$). Representative experiments shown. Complete supporting data are presented in Table S1.

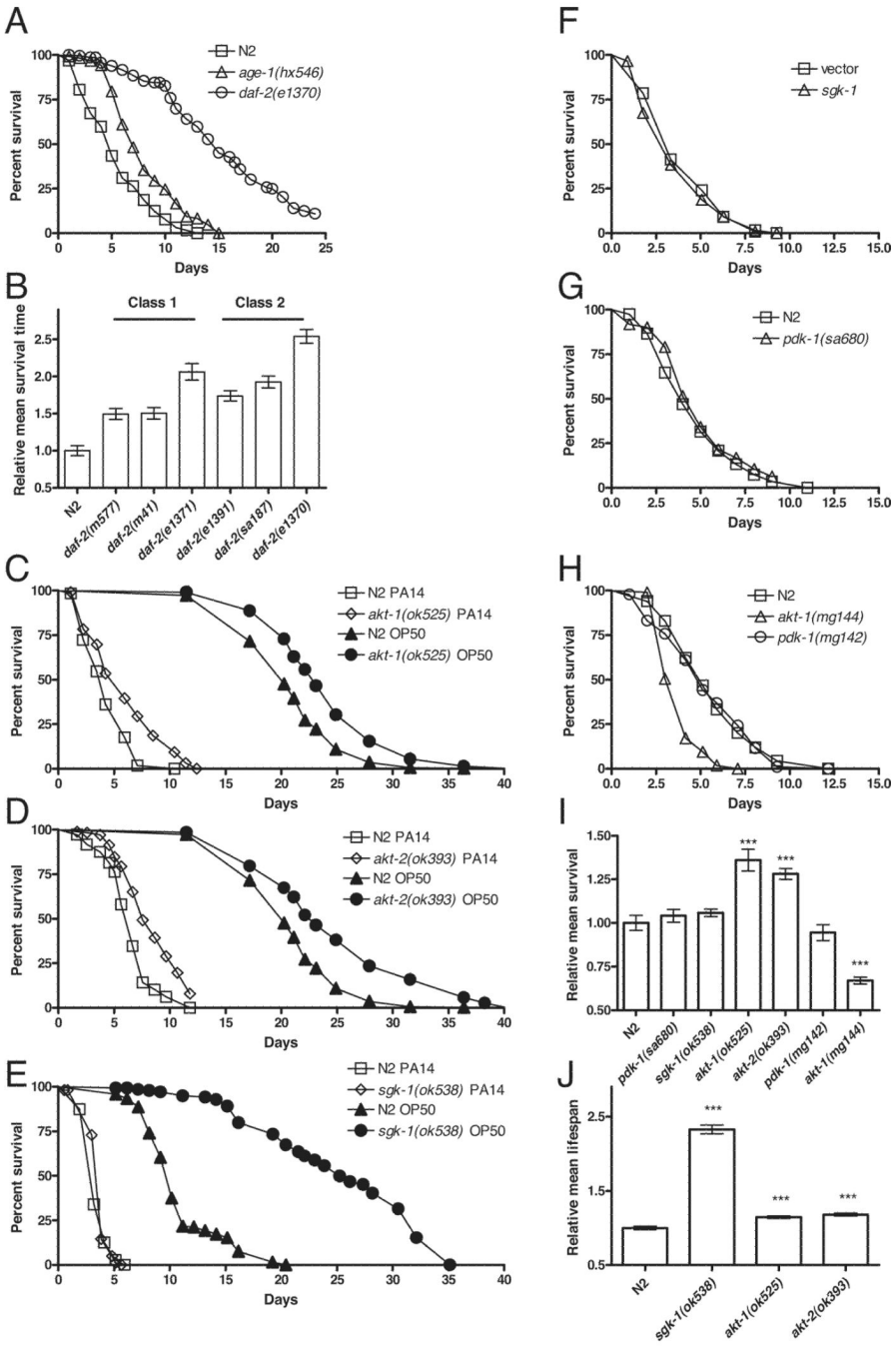

Figure 2. Aging and pathogen resistance are distinctly regulated by components of the insulin-like signaling pathway

(A) The enhanced pathogen resistance of long-lived *daf-2* and *age-1* mutants is observed in sterile animals. Survival of sterile *daf-2(e1370)*, *age-1(hx546)*, and N2 adults on PA14 over time is shown. The survival of each strain was significantly different in pairwise comparisons (logrank, $p < 0.0001$). (B) Hypomorphic *daf-2* mutants were resistant to PA14. *daf-2(m577)*, *daf-2(m41)*, *daf-2(1371)*, *daf-2(e1391)*, *daf-2(sa187)*, and *daf-2(e1370)* were more resistant to pathogen than N2 (logrank, $p < 0.0001$). Of the *daf-2* mutants tested, *daf-2(e1370)* was reproducibly the most resistant (logrank, $p < 0.0001$ in pairwise comparisons). Note that pathogen resistance varied more within than between phenotypic

classes among *daf-2* alleles (Gems *et al.* 1998). (C) *akt-1(ok525)* mutants were resistant to PA14 (logrank test, $p < 0.0001$; open symbols). They were also slightly, but significantly longer lived, with a mean lifespan of 115% compared to N2 (logrank $p < 0.0001$; closed symbols) when fed with UV-killed OP50 as 1-day old adults. (D) *akt-2(ok393)* mutants were resistant to PA14 (logrank test, $p < 0.0001$; open symbols). They were only slightly but significantly longer lived, with a mean lifespan of 118% compared to N2 (logrank $p < 0.0001$; closed symbols) when fed with UV-killed OP50 as 1-day old adults. (E) *sgk-1(ok583)* mutants were not significantly different than N2 in resistance to PA14 (logrank test, $p = 0.11$). *sgk-1(ok538)* mutants exhibited a mean lifespan of 230% compared to N2 (logrank, $p < 0.0001$). Lifespan of Glp N2 and *sgk-1(ok538)* fed with UV-killed OP50 starting as 1-day old adults is plotted. (F) The *sgk-1(ok538)* mutant has a strong developmental delay, which is substantially weaker with *sgk-1* RNAi knockdown. Thus, we used RNAi knockdown to assess the role of *sgk-1* in pathogen resistance without developmental timing as a confounding factor. RNAi knockdown of *cdc-25.1* and *sgk-1* was performed serially. *cdc-25.1* RNAi knockdown was performed maternally followed by egg lay to undiluted *sgk-1* RNAi bacteria. RNAi knockdown of *sgk-1* did not enhance pathogen resistance (logrank, $p = 0.27$). The efficacy of the induction of RNAi was confirmed by RNAi knockdown of *daf-16*, which enhanced susceptibility to pathogen (Table S1). Moreover, RNAi knockdown of *sgk-1* has been reported to extend lifespan to 160% of control levels (Hertweck *et al.* 2004). (G) *pdk-1(sa680)* mutants were also not significantly different than N2 in resistance to PA14 (logrank test, $p = 0.12$). (H) The pathogen resistance of gain-of-function mutants in *akt-1* and *pdk-1* mirrored the loss-of-function mutants. *akt-1(mg144)* was sensitive compared to N2 (logrank $p < 0.0001$), whereas *pdk-1(mg142)* was not significantly different from N2 (logrank $p = 0.6$). (I) Relative mean survival on PA14 for experiments shown in Figure 2. (J) Relative mean lifespan on UV-killed OP50 for experiments shown in Figure 2. All experiments used *cdc-25.1* RNAi Glp animals. Representative experiments shown. In panels C–E, representative lifespan and pathogen resistance assays were combined in the same plot to ease visual comparison. Complete supporting data are presented in Supplementary Table S1.

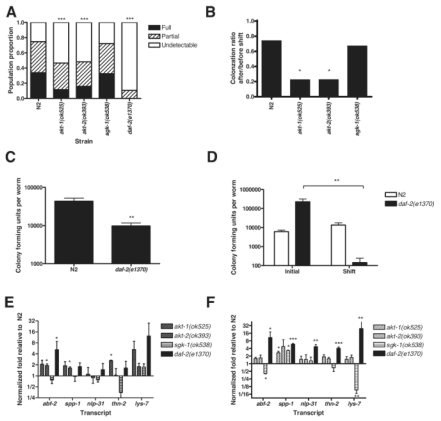

Figure 3. Resistance of *daf-2* pathway mutants is associated with reduced bacterial colonization, enhanced bacterial clearance, and increased immunity gene expression

(A) *akt-1(ok525)*, *akt-2(ok393)*, and *daf-2(e1370)* animals accumulated significantly less PA14-GFP in their intestine than N2 following a 24 h exposure (pairwise comparison Chi-square, $p < 0.0001$). No significant difference in bacterial accumulation was observed between *sgk-1(ok538)* and N2 populations (Chi-square, $p = 0.86$). For each strain, population proportion by degree of bacterial accumulation in the intestine was categorized as fully colonized, partially colonized, or undetectable colonization corresponding to averages of 4.22×10^4, 2.45×10^4, and 3.48×10^3 colony forming units of PA14-GFP, respectively. The pooled sample sizes are N=336 N2, 343 *akt-1(ok525)*, 328 *akt-2(ok393)*, 126 *sgk-1(ok538)*, and 28 *daf-2(e1370)*. (B) Pathogen clearance was enhanced in *akt-1(ok525)* and *akt-2(ok393)* compared to N2. The proportion of colonized worms before and after shift from PA14-GFP to *E. coli* OP50 was quantified, and the ratio of colonization after/before was plotted. The extent of clearance was significantly greater in *akt-1(ok525)* and *akt-2(ok393)* compared to N2 (log-linear analysis, $p < 0.05$) but not significantly different in *sgk-1(ok538)*. (C) Quantification of live bacteria from whole worm lysates of *daf-2(e1370)* and N2 following exposure to PA14 for 24 h. The CFU counts were significantly less in *daf-2* than N2 (t-test, $p = 0.009$). (D) Pathogen clearance was enhanced in *daf-2(e1370)* compared to N2. The initial PA14 CFUs obtained from *daf-2* and N2 worms exposed to PA14 for 24 h and 18 h, respectively, were compared to PA14 CFUs from worms obtained 24 h after the shift to OP50-1. While the CFUs from initial and shifted N2 populations were not significantly different (t-test, $p = 0.2$), the CFUs were reduced more than 1000 fold in *daf-2* worms after shift (t-test, $p = 0.01$). Use of equal initial exposure time produced comparable results after shift (data not shown). (E–F) Mean normalized expression level of immunity genes in *akt-1(ok525)*, *akt-2(ok393)*, *sgk-1(ok538)*, and *daf-2(e1370)* exposed to (E) OP50 or (F) PA14, plotted as fold relative to expression level in N2 exposed to PA14 on a $\log_2$ scale. Transcript levels were measured by quantitative RT-PCR and normalized to the average of 3 control genes. Mean and standard error of the mean from 2 to 4 independent experiments is shown. Pairwise comparisons to N2 were performed. (E) Antimicrobial gene expression is elevated in *akt-1(ok525)*, *akt-2(ok393)*, and *daf-2(e1370)* but not *sgk-1(ok538)*. (F) Each antimicrobial gene was expressed at significantly higher levels in *daf-2(e1370)* than N2 under infected conditions. Taken as a whole, the set of antimicrobials was expressed at higher levels in *daf-2(e1370)*, *akt-1(ok525)*, and *akt-2(ok393)* than N2 (binomial test, $p = 0.03$). Antimicrobial gene expression was mixed in *sgk-1(ok538)* mutants (binomial test, $p = 0.3$) with significantly lower levels of *abf-2* and *lys-7* but higher levels of *spp-1*. Error bars indicate standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

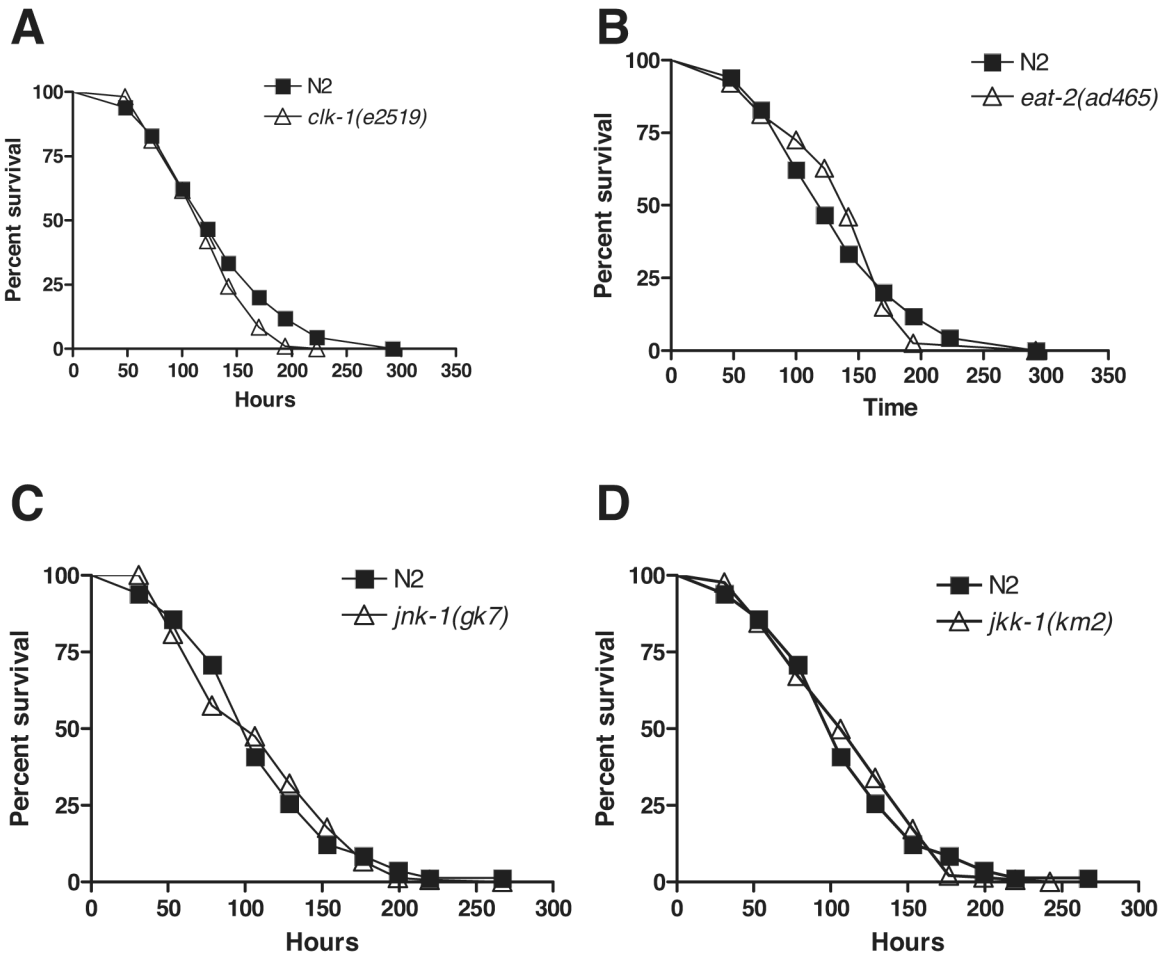

Figure 4. Pathogen resistance is not linked with lifespan extension in aging mutants

Other long and short-lived mutants do not have increased or decreased pathogen resistance. Neither *clk-1* (A) nor *eat-2* (B) were more resistant than N2 (logrank test, p = 0.02 and 0.7 respectively). *clk-1(e2519)* was not more sensitive than N2 as replicate experiments yield mean times to death from 92% to 104% relative to N2 (Supplemental Table S1). Likewise, neither *jnk-1(gk7)* (C) nor *jkk-1(km2)* (D) was more sensitive than N2 (logrank, p = 0.91 and 0.71 respectively). All experiments used *cdc-25.1* RNAi Glp animals. Representative experiments shown. Complete supporting data are presented in Supplementary Table S1.

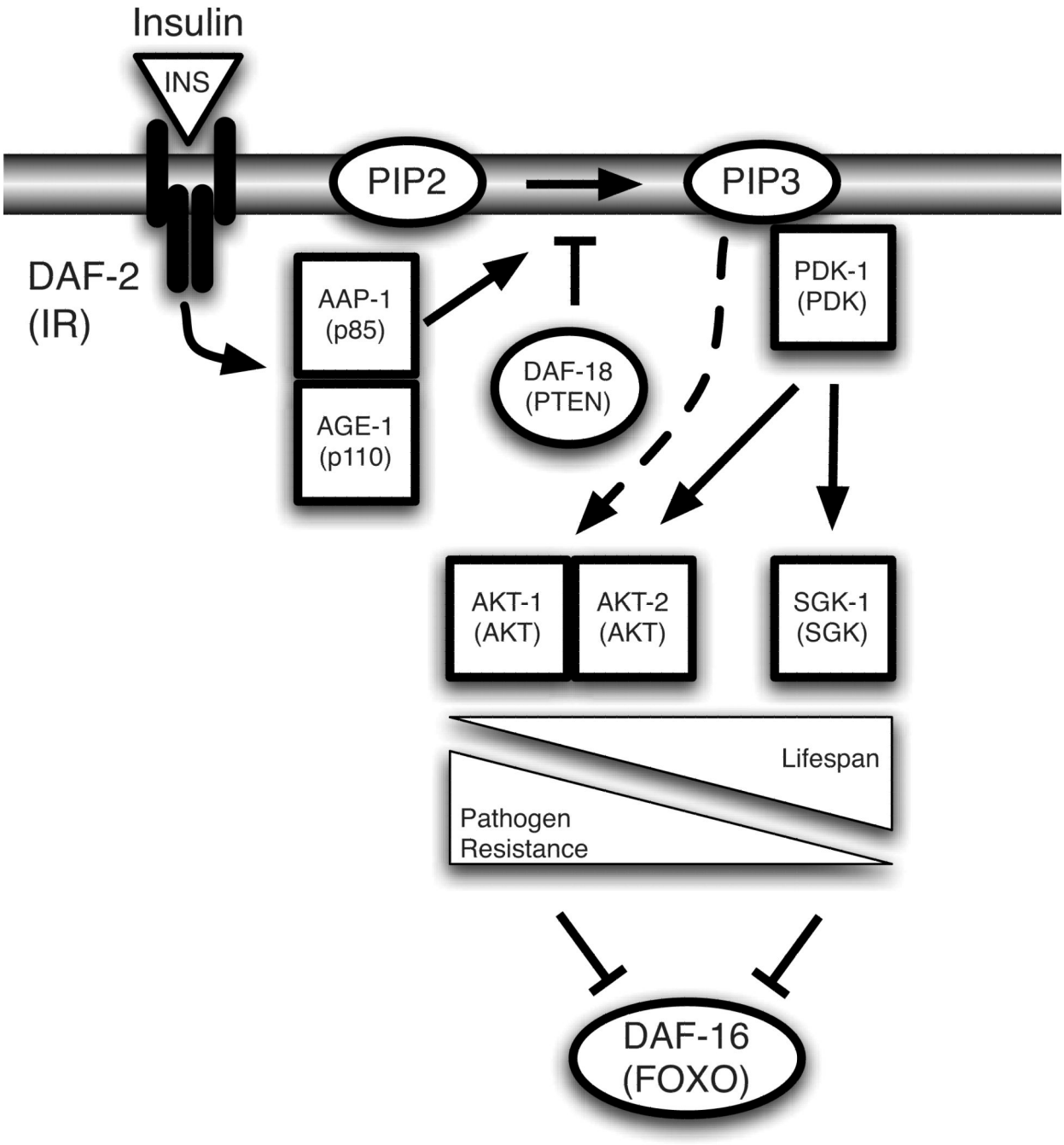

Figure 5. Model of the regulation of pathogen resistance and lifespan by insulin-like signaling

Activation of DAF-2 potentiates the generation of PIP3 by AGE-1, which in turn activates PDK-1. Activated PDK-1 leads to the phosphorylation of DAF-16 by AKT-1, AKT-2, and SGK-1. AKT-1 and AKT-2 contribute to the regulation of pathogen resistance and lifespan, whereas SGK-1 regulates lifespan but not pathogen resistance. A hypothetical PDK-1-independent input to AKT-1 and AKT-2 is also indicated. Model adapted from (Hertweck *et al.* 2004) with additional data from this report.