Cell, Vol. 121, 115–125, April 8, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.02.030

JNK Extends Life Span and Limits Growth by Antagonizing Cellular and Organism-Wide Responses to Insulin Signaling

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Summary

Aging of a eukaryotic organism is affected by its nutrition state and by its ability to prevent or repair oxidative damage. Consequently, signal transduction systems that control metabolism and oxidative stress responses influence life span. When nutrients are abundant, the insulin/IGF signaling (IIS) pathway promotes growth and energy storage but shortens life span. The transcription factor Foxo, which is inhibited by IIS, extends life span in conditions of low IIS activity. Life span can also be increased by activating the stress-responsive Jun-N-terminal kinase (JNK) pathway. Here we show that JNK requires Foxo to extend life span in *Drosophila*. JNK antagonizes IIS, causing nuclear localization of Foxo and inducing its targets, including growth control and stress defense genes. JNK and Foxo also restrict IIS activity systemically by repressing IIS ligand expression in neuroendocrine cells. The convergence of JNK signaling and IIS on Foxo provides a model to explain the effects of stress and nutrition on longevity.

Introduction

A number of heritable factors influence aging and promote the life expectancy of multicellular organisms. While genetic analysis has associated the activity of certain enzyme-encoding genes with increased life span (Guarente and Kenyon, 2000; Hekimi and Guarente, 2003; Holzenberger et al., 2003; Kenyon, 2001; Lin et al., 1997; Ogg et al., 1997; Tatar et al., 2003), the most prevalent systems that emerge from the search for longevity genes are signal transduction pathways that regulate gene expression in response to extracellular cues (Koubova and Guarente, 2003; Tatar et al., 2003). These pathways influence life span by acting both cell autonomously, governing cellular responses to local signals, as well as systemically, controlling physiology of the organism through endocrine mechanisms (Tatar et al., 2003). Insulin/IGF signaling (IIS), which regulates growth and energy storage of multicellular organisms, is firmly established as an effective and evolutionarily conserved signal transduction pathway that modulates aging and longevity.

Mutations in components of the IIS pathway significantly increase the life span of mice, *Drosophila*, and *C. elegans* (Bluher et al., 2003; Clancy et al., 2001; Holzenberger et al., 2003; Kenyon, 2001; Tatar et al., 2001). The effect of IIS on longevity may be linked to its well-documented influence on the metabolism. IIS promotes growth and energy storage when nutrients are abundant (Britton et al., 2002; Brogiolo et al., 2001; Datta et al., 1999; Garofalo, 2002; Ikeya et al., 2002; Kops et al., 2002; Rulifson et al., 2002; Saltiel and Kahn, 2001; Tatar et al., 2003). This function of IIS in growth control manifests itself in the decreased body size of loss-of-function mutants for IIS pathway components (Bohni et al., 1999; Oldham et al., 2002). Dietary restriction extends life span presumably through mechanisms that include reducing IIS activity, indicating that the regulation of growth and metabolism by IIS in accordance to nutritional conditions is a relevant modulator of longevity (Clancy et al., 2002).

IIS activity is systemically regulated by insulin-like peptides (ILPs). The *Drosophila* genome contains seven *Drosophila* ILP (*dilp*) genes, of which *dilp2* encodes a peptide that most closely resembles human insulin (Brogiolo et al., 2001). Dilp2 is secreted from a small cluster of neuroendocrine cells in the brain, the insulin-producing cells (IPCs, Ikeya et al. [2002]; Rulifson et al. [2002]). Thus, IPCs are functionally analogous to mammalian pancreatic β cells, and accordingly they are required for carbohydrate homeostasis in the fly's hemolymph (Rulifson et al., 2002). The central role of Dilps in the systemic regulation of IIS activity has been demonstrated in ablation experiments. When IPCs are specifically eliminated, developmental delays and growth retardation ensue. Late-life mortality is decreased in these animals, suggesting that life span is regulated by Dilps secreted from these cells (Rulifson et al., 2002; Wessells et al., 2004).

In their target tissues, Dilps activate the insulin-like growth factor receptor (InR; Britton et al. [2002], Rulifson et al. [2002]). InR signals through Chico, a homolog of the mammalian insulin receptor substrates 1–4 (IRS 1–4; Bohni et al. [1999]), PI3 kinase (PI3K), and Akt. The phosphatase PTEN antagonizes PI3K activity and, hence, IIS (Goberdhan and Wilson, 2003). IIS affects gene expression by inactivating the Forkhead Box O transcription factor Foxo. This involves Akt-mediated phosphorylation and subsequent cytoplasmic retention of Foxo, resulting in downregulation of its target genes (Accili and Arden, 2004; Hekimi and Guarente, 2003; Kenyon, 2001). The activation of Foxo in conditions of reduced IIS activity affects growth and longevity. The Foxo homolog in *C. elegans*, Daf-16, for example, is essential for the longevity phenotype of IIS loss-of-function mutants (Hekimi and Guarente, 2003; Kenyon, 2001; Lin et al., 1997; Ogg et al., 1997; Tatar et al., 2003). *Drosophila* Foxo (DFoxo) is similarly required for growth repression in IIS mutant flies (Jünger et al., 2003; Puig et al., 2003) and extends life span when overexpressed (Giannakou et al., 2004; Hwangbo et al., 2004).

There is a striking but not completely understood correlation between longevity and oxidative stress re-

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sistance in IIS mutants (Beckman and Ames, 1998; Guarente and Kenyon, 2000). In worms, flies, and mice, genetic alterations that reduce IIS and increase the activity of Foxo result in tolerance against oxidative insults (Guarente and Kenyon, 2000; Hekimi and Guarente, 2003; Holzenberger et al., 2003; Kenyon, 2001; Lin et al., 1997; Ogg et al., 1997; Tatar et al., 2003). Conversely, loss-of-function mutants of *dfoxo* are viable but exhibit reduced tolerance to oxidative stress (Jünger et al., 2003; Puig et al., 2003). Studies in mammalian cell culture have further shown that Foxo activity protects against stress-inflicted cellular damage (Kops et al., 2002; Tran et al., 2002). Similar to starvation, oxidative stress causes Foxo to translocate to the nucleus, where it stimulates the expression of target genes that encode proteins involved in stress protection, damage repair, and cell cycle arrest (Hsu et al., 2003; Murphy et al., 2003). Foxo thus integrates both nutritional and stress signals to regulate growth, cell proliferation, and stress tolerance (Accili and Arden, 2004).

While it is well established that nutritional cues are relayed to Foxo by IIS (Accili and Arden, 2004; Jünger et al., 2003), it is less clear how the transcription factor becomes activated when cells or organisms encounter environmental stress (Accili and Arden, 2004; Brunet et al., 2004; Henderson and Johnson, 2001; Lin et al., 2001). In *C. elegans*, nuclear translocation of Daf-16/Foxo in response to heat stress depends on the PTEN homolog Daf-18, suggesting that such insults might be sensed by a mechanism that downregulates IIS upstream of PTEN (Lin et al., 2001). However, in mammalian cells exposed to oxidative stress, the Akt substrate sites on Foxo, which are believed to be the conduit from IIS, do not change their phosphorylation state under conditions that induce Foxo target gene expression (Brunet et al., 2004). Additional signaling inputs that antagonize IIS and/or activate Foxo in response to stress may thus exist.

The evolutionarily conserved Jun-N-terminal Kinase (JNK) pathway is a plausible candidate for an additional input into Foxo regulation. It is activated in response to a variety of environmental insults, including UV irradiation and oxidative stress (Davis, 2000; Kockel et al., 2001; Stronach and Perrimon, 1999; Weston and Davis, 2002). In *Drosophila*, JNK confers tolerance to oxidative stress and extends life span by inducing a protective gene expression program (Wang et al., 2003). The signaling events downstream of JNK that mediate this effect are not presently known.

JNK is part of a mitogen-activated protein kinase (MAPK) cascade that in *Drosophila* consists of the JNK kinase (JNKK) Hemipterous (Hep) and the JNK Basket (Bsk; Davis [2000], Kockel et al. [2001], Stronach and Perrimon [1999], Weston and Davis [2002]). Bsk phosphorylates transcription factors and thereby induces changes in gene expression. JNK signaling strength and duration is dampened by a negative feedback loop mediated by one of its target genes, *puckered* (*puc*), which encodes a JNK-specific dual specificity MAPK phosphatase. Reduction of the *puc* gene dose (in heterozygotes for *puc* loss-of-function alleles) increases basal levels of JNK signaling, resulting in increased stress tolerance and longevity (Wang et al., 2003; Zeitlinger and Bohmann, 1999).

The induction of gene expression programs for stress defense and damage repair by activated Foxo suggests that it might influence longevity by mechanisms that act at the cellular level. Consistent with this notion, a recent study shows that tissue-autonomous functional decline and somatic senescence in the aging *Drosophila* heart is decreased when IIS is suppressed or Foxo is overexpressed in just this tissue (Wessells et al., 2004). Remarkably, flies in which premature heart failure is thus prevented do not live longer than wild-type controls, suggesting that Foxo activation or IIS reduction has to occur in multiple tissues to noticeably increase life span (Wessells et al., 2004). Such a global response might rely on endocrine mechanisms, conceivably modulation of systemic Dilp levels, to influence senescence of the organism in a coordinated fashion. In line with such a model, overexpression of Foxo, when restricted to tissues with endocrine functions, such as the fatbody of *Drosophila*, or neuronal tissue or intestine of *C. elegans*, extends life span (Giannakou et al., 2004; Hwangbo et al., 2004; Libina et al., 2003). The exact signaling events that cause this effect are unknown.

Here, we report that the life span of flies is influenced by an antagonistic relationship between JNK signaling and IIS. We show that JNK promotes nuclear translocation of Foxo and induces the expression of Foxo-dependent stress response genes that conceivably promote cell-autonomous stress defense and damage repair. Consistent with this finding, we demonstrate that Foxo is required for JNK to extend life span. Interestingly, we find that JNK also antagonizes IIS systemically by activating Foxo and downregulating the expression of *dilp2* in IPCs. Our studies thus identify cellular and endocrine mechanisms by which JNK and IIS determine life span in *Drosophila*.

Results

dfoxo Is Required for JNK-Mediated Life Span Extension

Populations of flies in which JNK is mildly activated due to heterozygosity for *puc* display a significantly longer average life span than isogenic wild-type controls (Wang et al., 2003). To examine if these effects might be mediated by Foxo, we tested whether they could be suppressed when the gene dose of *dfoxo* was reduced (Figure 1). In a wild-type background, heterozygosity for either of two *dfoxo* mutant alleles had minor effects on life span. However, the long-lived phenotype of *puc*/+ animals was reverted to wild-type when one gene dose of *dfoxo* was eliminated (Figure 1; this effect was observed in both female [Figure 1A] and male [Figure 1B] flies). We conclude that DFoxo plays a critical role in JNK-mediated life span extension. A possible explanation for this effect would be the regulation of Foxo activity by a JNK-dependent mechanism. To test this idea, we proceeded to examine whether JNK signaling influences Foxo activity in vivo.

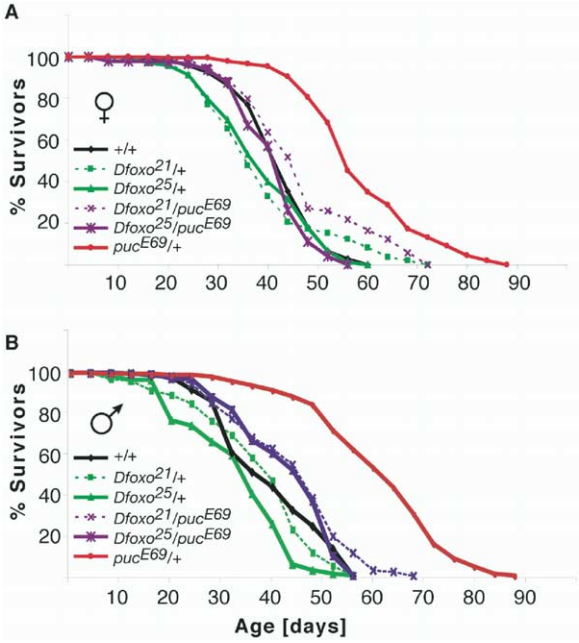

Figure 1. Life Span Extension by JNK Requires *dfoxo*

(A and B) Survival of fly populations at 25°C as recorded from individual cohorts of about 100 flies. Heterozygosity for puc^{E69} extends life span compared to coisogenic wild-type controls due to increased JNK signaling activity (see Wang et al. [2003]). Heterozygosity for puc^{E69} does not extend life span in $dfoxo^{21}$ or $dfoxo^{25}$ heterozygous flies. $dfoxo^{21}$ and $dfoxo^{25}$ are loss-of-function alleles of *dfoxo* (point mutations introducing stop codons at W95 and W124, respectively; Jünger et al. [2003]). Note that heterozygosity for $dfoxo^{21}$ or $dfoxo^{25}$ does not affect life span in wild-type backgrounds. Fly stocks were made coisogenic by backcrossing (ten generations). Survival data from separate experiments are combined here for clarity. Genotypes and resulting population sizes are as follows: (A) Females: *yw*/+;; $dfoxo^{21}$/+ n = 257. *yw*/+;; $dfoxo^{25}$/+ n = 194. *yw*/+;; ry^{506}/+ n = 330. *yw*/+;; $dfoxo^{21}/puc^{E69}$, ry^{506} n = 224. *yw*/+;; $dfoxo^{25}/puc^{E69}$, ry^{506}/+ n = 145. *yw*/+;; puc^{E69}, ry^{506}/+ n = 276. (B) Males: *yw*/Y;; $dfoxo^{21}$/+ n = 214. *yw*/Y;; $dfoxo^{25}$/+ n = 230. *yw*/Y;; ry^{506}/+ n = 330. *yw*/Y;; $dfoxo^{21}/puc^{E69}$, ry^{506} n = 255. *yw*/Y; $dfoxo^{25}/puc^{E69}$, ry^{506} n = 144. *yw*/Y;; puc^{E69}, ry^{506}/+ n = 223. Statistical analysis was performed using the log rank test. For *puc*/+ versus *dfoxo*/*puc*, $p < 0.001$.

JNK Signaling Modulates *dfoxo*-Dependent Phenotypes in the Eye

To investigate potential effects of JNK signaling on Foxo function, we examined whether changes in JNK activity would influence DFoxo-mediated phenotypes in vivo (Figure 2). Overexpression of DFoxo in the developing *Drosophila* eye causes externally visible defects (Figure 2A; compare to wild-type eyes, Figure 2F; Jünger et al. [2003]). These defects were significantly enhanced when JNK activity was mildly elevated by coexpression of the JNK kinase Hep (Figure 2B). Conversely, blocking JNK signaling with a dominant-negative form of the *Drosophila* JNK Basket (Bsk^{DN}; Figure 2C; Weber et al. [2000]) reversed the effects of DFoxo expression and mostly restored the wild-type appearance of the eye. Overexpression of neither Hep nor Bsk^{DN} in an otherwise wild-type eye induced any discernible phenotypes (Figures 2D-2F). The modulation

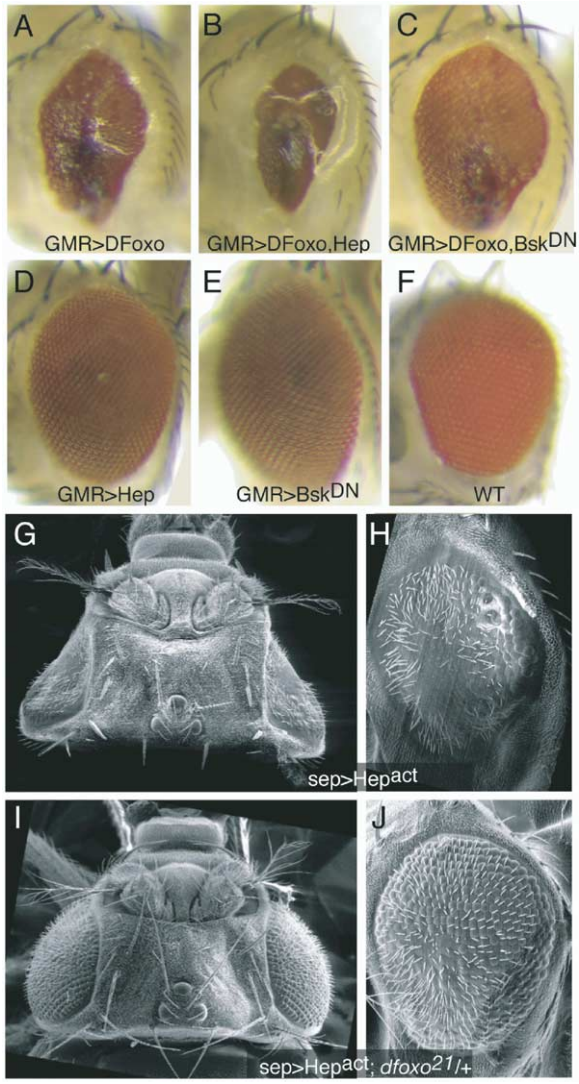

Figure 2. JNK Signaling Modulates DFoxo Activity

(A–F) JNK signaling levels influence the severity of DFoxo gain-of-function phenotypes. The eye phenotype observed in animals overexpressing DFoxo under the control of the eye-specific Gal4 driver GMR ([A], compare to wild-type eyes, [F]) is enhanced when Hep (B) and rescued when Bsk^{DN} (C) are cooverexpressed. Expression of neither Hep nor Bsk^{DN} alone leads to eye defects under these conditions (D and E).

(G–J) JNK gain-of-function phenotypes in the eye require *dfoxo*. Constitutive activation of JNK in photoreceptors and cone cells by overexpressing Hep^{act} under the control of Sep-Gal4 leads to severe developmental defects (G and H). These defects are rescued in *dfoxo* heterozygous conditions (I and J).

All flies were reared under identical nutritional conditions at 25°C.

of the DFoxo-induced eye phenotype by JNK signaling is reminiscent of the effects of IIS or starvation in similar experiments. Jünger et al. have shown that the phenotype induced by ectopic Foxo is suppressed in IIS gain-of-function conditions and, conversely, aggravated by starvation (Jünger et al., 2003). These data are consistent with the idea that JNK and IIS can both influence the activity of Foxo and suggest that Foxo

might be required to mediate aspects of JNK-dependent phenotypes in the eye. To test this latter hypothesis, we asked whether *dfoxo* activity was required for the manifestation of JNK gain-of-function phenotypes in the eye. Expression of a constitutively active form of Hep (Hep^{act}; Weber et al., 2000) in the developing eye imaginal disc results in severe malformations of the adult eye (Figures 2G and 2H; as opposed to overexpression of the phenotypically neutral wild-type protein, Figure 2E). This effect was suppressed in flies heterozygous for a *dfoxo* loss-of-function allele (Figures 2I and 2J). The requirement of Foxo for Hep-induced phenotypes indicates that it is an important mediator of JNK-induced cellular effects, at least under gain-of-function conditions.

JNK Represses IIS-Induced Growth and Promotes Nuclear Translocation of Foxo

To assess how JNK signaling might affect IIS-induced biological responses at the cell level, we turned to the larval fatbody. In response to nutritional cues, growth of endoreduplicating fatbody cells is stimulated by IIS (Britton et al., 2002).When IIS is artificially activated by overexpression of dInR in fatbody cell clones, dramatic overgrowth ensues (Figures 3A, 3D, and 3G; Britton et al. [2002]). As expected from cell culture studies (Jünger et al., 2003; Puig et al., 2003), no nuclear DFoxo is detectable in these cells (Figure 3D). To test whether JNK activation could activate Foxo and repress these effects of high IIS activity, we cooverexpressed InR with Hep^{act}. Strikingly, overgrowth was significantly reduced and DFoxo was relocalized to the cell nucleus in these cells (Figures 3B, 3E, and 3H). Overexpression of Hep^{act} alone had only minor effects on cell size (Figures 3C, 3F, and 3I). Consistent with our observations in the eye (Figure 2), these findings support the notion that JNK signaling can stimulate Foxo activity and antagonize IIS-mediated overgrowth cell autonomously.

JNK Regulates the Expression of Foxo Target Genes

The nuclear translocation of DFoxo in response to JNK signaling suggests that transcriptional targets of DFoxo would be induced upon JNK activation. Based on studies in mammalian cells, *C. elegans*, and *Drosophila*, Foxo-dependent gene regulation has at least two important functions. One is to arrest cell growth; the other is to prevent stress-induced cellular damage (Hsu et al., 2003; Murphy et al., 2003).

A target gene of DFoxo that has been reported to mediate growth control effects is *thor*, which encodes the *Drosophila* homolog of eIF4E binding protein (4E-BP) (Jünger et al., 2003; Puig et al., 2003). Its activity as a translational repressor is expected to suppress cell and organ growth when IIS is inactive (Miron et al., 2001; Puig et al., 2003). To test the idea that Foxo-dependent gene expression is controlled by the JNK pathway, we measured the levels of *thor* mRNA in embryos of different genotypes. Using RT-PCR, we found that *thor* expression was increased in JNK gain-of-function conditions and decreased when JNK activity was suppressed by a dominant-negative form of Bsk (Figure 4A).

To find out whether *thor* would also be regulated by oxidative stress in adult flies, we measured *thor* transcript levels in flies that were exposed to the oxygen radical-generating compound Paraquat using real-time RT-PCR. As observed for other JNK-responsive genes (Wang et al., 2003), *thor* was induced in response to Paraquat. This induction was dependent on *dfoxo* and *hep* gene function (Figure 4B), further supporting the notion of Foxo acting as a nuclear effector of JNK.

We next tested whether JNK would also stimulate expression of DFoxo target genes that are involved in stress protection. In *C. elegans*, Daf-16/Foxo influences life span by inducing expression of the small heat shock proteins Hsp-16.1, Hsp-12.6, Hsp-16.49, and Hsp-16.11 (Hsu et al., 2003; Murphy et al., 2003). Small heat shock proteins (sHsps) enhance the survival of cells exposed to oxidative stress (Arrigo, 1998; Welsh and Gaestel, 1998) and may delay aging by preventing the accumulation of protein aggregates (Hsu et al., 2003; Murphy et al., 2003). The *Drosophila shsp* homolog *l(2)efl* is induced in response to oxidative stress (Landis et al., 2004). We asked whether JNK and Foxo may regulate the expression of *l(2)efl* and found that it is responsive to JNK signaling and DFoxo in embryos (Figure 4A) and adults (Figure 4C; real-time RT-PCR). We also examined whether the induction of *l(2)efl* by JNK in embryos would require normal *foxo* gene function. In embryos in which the *dfoxo* gene dose was reduced by half, *l(2)efl* induction by Hep^{act} expression was significantly reduced (Figure 4D). This result supports a role for Foxo in the control of protective gene expression by JNK. The induction of *l(2)efl* by this mechanism can be expected to increase oxidative stress resistance (Arrigo, 1998; Welsh and Gaestel, 1998) and possibly longevity. To test this idea, we measured life span in populations of flies that overexpress *l(2)efl* transgenes (Figures 4E and 4F). We observed significant extension of life span compared to isogenic control lines when *l(2)efl* was expressed ubiquitously (using the ubiquitous Gal4 driver armG4; Figure 4E), indicating that *l(2)efl* is an important mediator of JNK and Foxo in the control of life span. Interestingly, neuronally restricted *l(2)efl* overexpression (using the panneuronal driver elavG4; Figure 4F) was sufficient to extend life span. This effect suggests that *l(2)efl* might specifically protect neurons against age-related damage. The ability of sHsps to prevent the accumulation of protein aggregates (Hsu et al., 2003), which is harmful to neurons, may explain this observation.

Interestingly, several other JNK-responsive genes with putative function in stress protection (Wang et al., 2003) are regulated by DFoxo (among them *hsp68*, *fax*, *fer1HCH*, and *CG14207*; O. Puig, personal communication). Furthermore, overexpression of Hsp68 also extends life span in *Drosophila* (Wang et al., 2003), indicating that the transcriptional induction of this group of stress-response genes may account, at least in part, for the life span extension by JNK and DFoxo.

Systemic Repression of IIS by JNK and Foxo

The data described above show that JNK and IIS regulate Foxo in opposite ways. This finding is consistent with the different effects of the two pathways on lon-

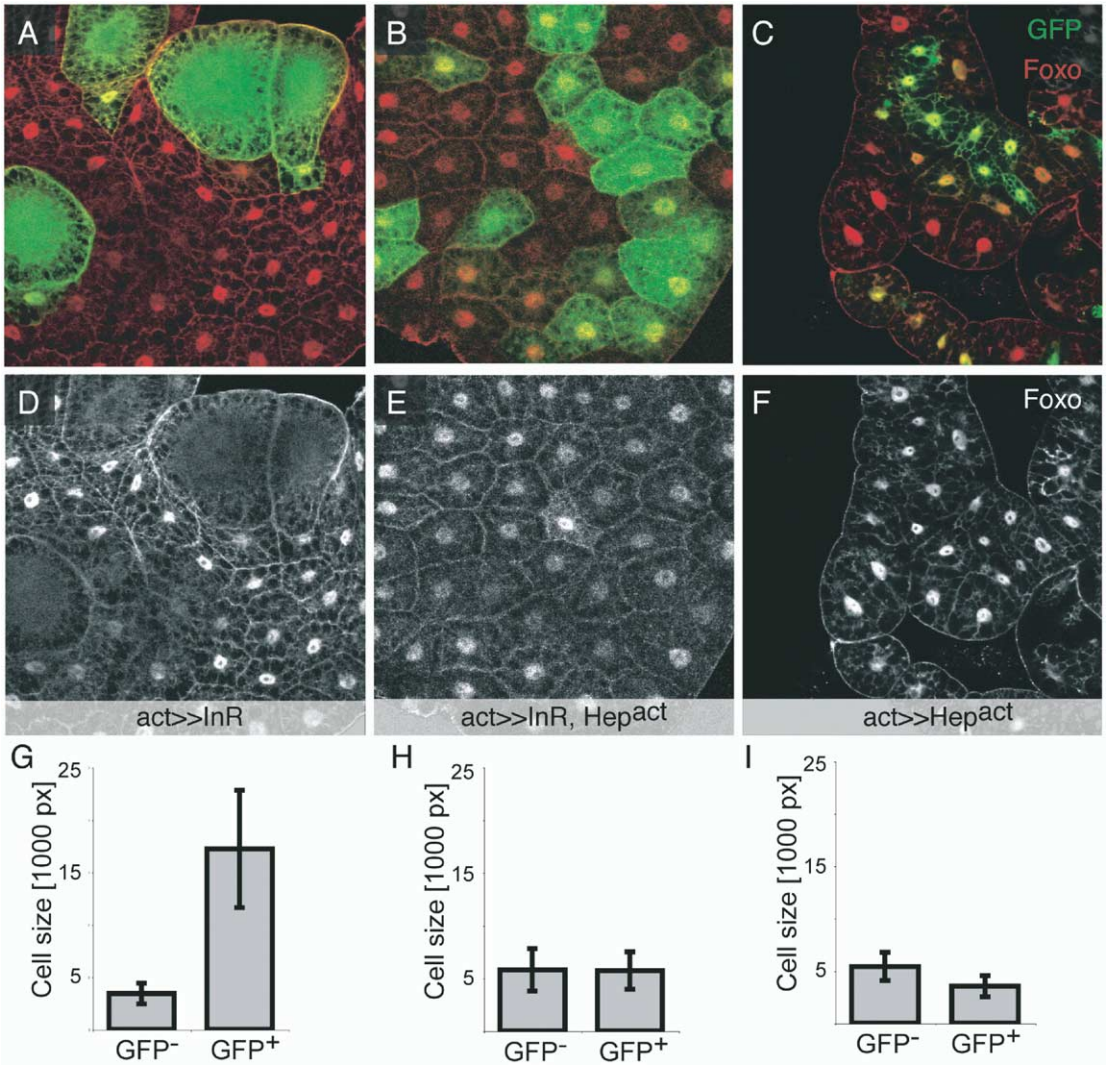

Figure 3. JNK Antagonizes IIS and Promotes DFoxo Nuclear Translocation

(A–I) Third instar larval fatbody cell clones expressing UAS-linked transgenes under the control of the *actin* promoter. Clones are marked by GFP (green). DFoxo protein is visualized with anti-DFoxo antibody (Puig et al. [2003]; red in [A]–[C]; white in [D]–[F]). Cell sizes were quantified using image processing ([G–I]; each bar is the size average and standard deviation from 15 cells from three different larvae). In clones overexpressing DInR, fatbody cells overgrow and DFoxo is excluded from the nucleus (A, D, and G). Hep^{act} cooverexpression reverts overgrowth and relocalizes Foxo to the nucleus (B, E, and H). No obvious defects are observed in cells overexpressing Hep^{act} alone (C, F, and I). InR expression levels, determined by RT-PCR, are identical in (A) and (B) (data not shown).

gevity and stress resistance. We wondered whether the antagonism between JNK and IIS in the regulation of transcriptional stress defense programs at the level of individual cells would be sufficient to explain the longevity of flies in which JNK is activated, or whether global, cell nonautonomous effects might have to be invoked.

In previous studies, we have found that increasing JNK activity in a manner that is restricted to neurons is sufficient to promote stress tolerance and extend life span of the whole organism (Wang et al., 2003). It is unclear whether this effect was due to the specific protection of neurons against oxidative damage or whether JNK activation in neurons might induce a humoral response that would regulate longevity by a systemic mechanism. Interestingly, we found that, in the brain of adult flies, high levels of JNK activity can be detected specifically in the IPCs (Figures 5A and 5B). This observation suggests an important function for JNK in these cells, possibly in the regulation of Dilp expression. As it has been suggested that DFoxo affects physiology and life span nonautonomously by reducing Dilp2 production in the IPCs (Hwangbo et al., 2004), we asked whether JNK would contribute to this regulation of *dilp2* expression. Using RT-PCR, we found that *dilp2* transcript levels were significantly reduced in the head of *puc* heterozygotes as well as in flies in which Hep^{act} was specifically overexpressed in IPCs (Figure 5C; dilp2-Gal4 directs expression of UAS-linked transgenes specifically to IPCs, Figure 5D; Rulifson et

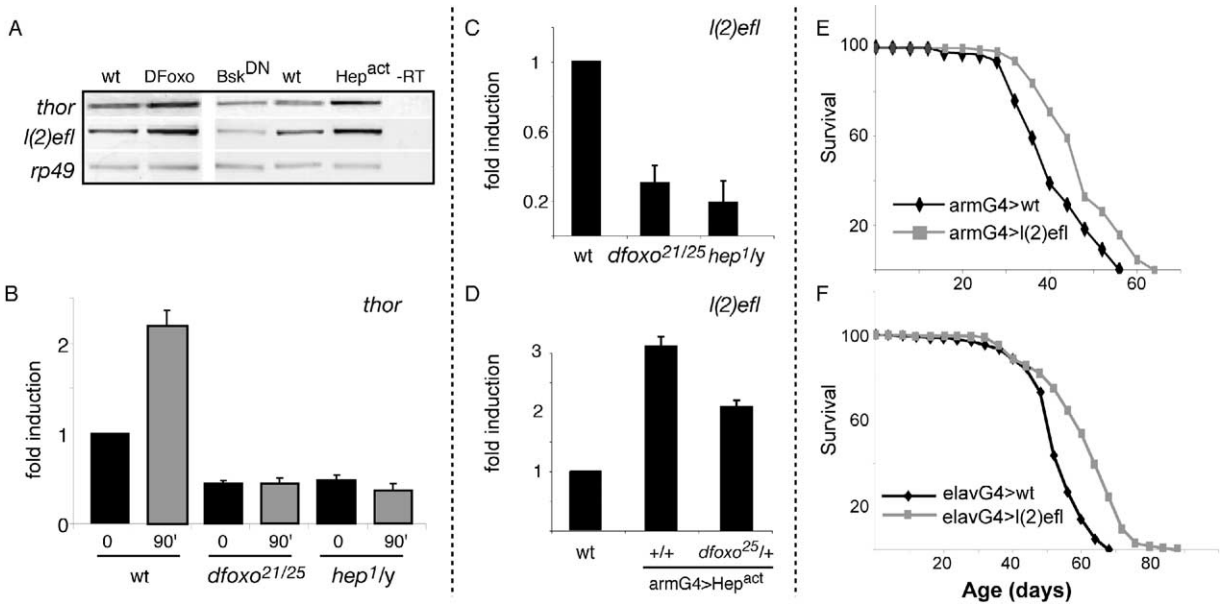

Figure 4. Coordinated Regulation of Gene Expression by JNK Signaling and DFoxo

(A) RT-PCR measuring *thor* and *l(2)efl* mRNA levels in embryos overexpressing DFoxo (under the control of armadillo Gal4; arm > DFoxo) and in embryos with repressed (arm > Bsk^{DN}) or activated (arm > Hep^{act}) JNK signaling. Both genes are coregulated by JNK signaling and DFoxo. *ribosomal protein 49* (*rp49*) transcript levels serve as internal controls.

(B) Induction of *thor* mRNA in 1-day-old adult males after Paraquat exposure (0, mock exposure; 90′, 90 min exposure; $p = 0.007$, Student's t test) as measured by real-time RT-PCR. Induction is not detectable in hep^{1} hemizygous and $dfoxo^{21}/dfoxo^{25}$ transheterozygous animals. Transcript levels are normalized to *rp49* levels and to mock exposure in wild-type animals. Note also the reduced levels of *thor* in mock-exposed mutant animals compared to mock-exposed wild-type flies ($p < 0.005$).

(C) *l(2)efl* expression is reduced in hep^{1} hemizygous and $dfoxo^{21}/dfoxo^{25}$ transheterozygous 1-day-old adult males as compared to wild-type control animals ($p < 0.008$).

(D) The induction of *l(2)efl* mRNA by JNK in embryos is significantly reduced in a *dfoxo* heterozygous background ($p < 0.01$; genotypes: armGal4/+; +/+ |$UASHep^{act}$/armGal4;+/+| $UASHep^{act}$/armG4;$dfoxo^{25}$/+). Real time RT-PCR normalized to *rp49* levels and to *l(2)efl* levels in wild-type controls.

(E and F) Ubiquitous (armG4; [E]) or panneuronal (elavG4; [F]) overexpression of *l(2)efl* is sufficient to extend life span. Fly stocks were backcrossed into the w^{1118} genetic background. Survival data from separate experiments are combined here for clarity. Genotypes and resulting population sizes are as follows (only males were scored): *w/Y;armG4/+;*UASl(2)efl/+; n = 339. *w/Y;armG4/+;+/+* n = 340. *w,elav*^{C155}*G4/Y;;*UASl(2)efl/+ n = 382. *w,elav*^{C155}*G4/Y;+/+* n = 322. Statistical analysis was performed using the log rank test. For all cases, $p < 0.001$.

al. [2002]). This repression was dependent on functional *dfoxo*, as it was reduced in a *dfoxo* mutant background (Figure 5C), indicating that Foxo acts downstream of JNK to repress *dilp2* expression in IPCs. IPCs were readily detectable in flies expressing Hep^{act} under the control of dilp2-Gal4, confirming that downregulation of *dilp2* expression was not due to IPC depletion (Figure 5E). Further supporting a stimulatory function of JNK on Foxo in IPCs, we found that expression of Hep^{act} in these cells resulted in nuclear translocation of Foxo (Figures 5F–5I). JNK thus appears to directly regulate Foxo function in IPCs.

In agreement with the known function of Dilps as systemic regulators of growth (Ikeya et al., 2002; Rulifson et al., 2002), we observed a significant Foxo-dependent decrease in body size of flies overexpressing Hep^{act} specifically in IPCs (Figures 5J and 5K). The growth-retarding effect of IPC-restricted Hep^{act} expression was reproduced when Foxo itself was overexpressed in the same cells (Figures 5L and 5M). This finding supports a role for Foxo as a JNK effector in this context.

Next, we asked whether the systemic effect of IPC-specific JNK activation might be sufficient to extend life span of the organism. To increase JNK activity moderately in IPCs, we overexpressed the wild-type form of Hep using the dilp2-Gal4 driver (Figure 6; Rulifson et al. [2002]). This moderate increase of JNK activity in IPCs does not notably affect organism growth (data not shown) but extends life span significantly in both male and female flies (Figures 6A and 6B). Consistent with our model, *dilp2* expression in these animals is moderately reduced (Figure 6C). This result supports a role for JNK-mediated repression of *dilp2* expression in the control of longevity.

Discussion

Our data suggest Foxo as a convergence point for IIS and JNK signaling. Through its responsiveness to these two pathways, Foxo is well positioned to integrate information about environmental stress and nutrient availability and to elicit appropriate biological responses. Such a system would ensure that growth could proceed in an unrestrained manner when energy resources are available and the cell is not exposed to external insults (IIS is active, JNK is off, and Foxo is

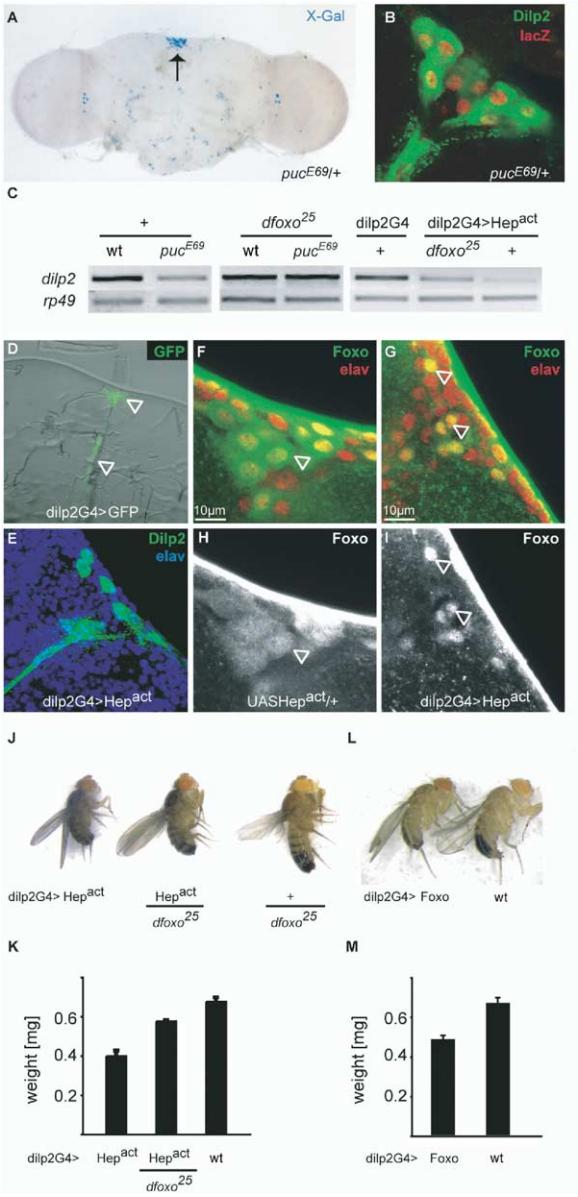

Figure 5. JNK Represses Insulin-Like Peptide Production and Affects Growth

(A and B) JNK is active in insulin-producing cells (IPCs) in the brain of adult flies carrying the JNK responsive *puc*^{E69} nuclear β-galactosidase reporter (Martin-Blanco et al., 1998). X-Gal (A) and immunostaining ([B], anti-β-galactosidase in red, single confocal section) in brains from *puc*^{E69}/+ adult flies reveal JNK activity in IPCs (arrow in [A] and labeled with anti-Dilp2 antibody, green, in [B]).

(C) *dilp2* expression is reduced in *puc*^{E69} heterozygotes and in flies expressing Hep^{act} specifically in IPCs (using the dilp2-Gal4 driver; Rulifson et al. [2002]). This repression requires Foxo activity. Genotypes (from left to right): *ry*^{506}/ *ry*^{506} |*puc*^{E69}, *ry*^{506}/*ry*^{506}| *ry*^{506}/*dfoxo*^{25} |*puc*^{E69}, *ry*^{506}/*dfoxo*^{25}| dilp2G4/+ |dilp2G4/UASHep^{act}; *dfoxo*^{25}/+| dilp2G4/UASHep^{act}; +/+. RT-PCR detecting *dilp2* transcripts from cDNA derived from heads of 1-day-old male flies. *rp49* transcript levels serve as internal controls.

(D) Dilp2-Gal4 drives expression of UAS-linked transgenes specifically in IPCs of the adult brain (brain of 1-day-old male fly; GFP in green; image is a composite of transmission and GFP fluorescence recorded on a confocal microscope). IPC cell bodies and axons are visible (arrowheads). Genotype, dilp2-Gal4/UAS-GFP.

repressed). However, in situations of low food availability or an adverse environment, IIS would cease to signal, or JNK would be activated, resulting in translocation of Foxo to the nucleus. The ensuing Foxo-induced gene expression has several effects at the cell as well as the organism level and is likely to counteract premature senescence. The induction of genes such as *thor* can reduce cell growth (Miron et al., 2001), presumably to limit the cell's anabolic expenses in adverse situations. Other target genes, such as the small heat shock protein *l(2)efl*, are expected to have a direct role in allaying damage inflicted by environmental insults and may prevent the accumulation of toxic protein aggregates (Hsu et al., 2003; Murphy et al., 2003). The suppression of *dilp2* expression by JNK and Foxo in IPCs, on the other hand, is likely to control growth, metabolism, and stress responses systemically by downregulating IIS in all responsive tissues in a coordinated fashion.

Endocrine Regulation of Aging by JNK in IPCs

The interaction between JNK and Foxo is thus expected to influence stress tolerance and life span at two levels. In peripheral tissues, JNK activates Foxo and prevents senescence cell-autonomously. Such a mechanism is exemplified by the recent finding that Foxo overexpression prevents age-dependent decline of cardiac performance (Wessells et al., 2004). Systemic control of IIS by JNK-mediated activation of Foxo in IPCs, on the other hand, would serve to coordinate cellular responses to changes in the environment throughout the organism. Our data indicate that this latter mechanism plays a significant role in the regulation of life span by JNK and Foxo. The identification of this endocrine function of JNK/Foxo signaling supports and extends the proposed role of JNK signaling on longevity and demonstrates a role for IPCs in life span regulation. In addition to controlling growth and metabolism

(E) Hep^{act} expression does not cause apoptosis in IPCs. Hep^{act}-expressing IPCs in the adult brain detected using anti-Dilp2 antibody (green). Neuronal nuclei detected with anti-Elav antibody (blue).

(F–I) Hep^{act} expression in IPCs results in nuclear translocation of DFoxo. Endogenous Foxo detected using anti-DFoxo antibody (green in [F] and [G], white in [H] and [I]; Puig et al. [2003]). Cell nuclei are visualized using anti-Elav antibody (red). In wild-type cells (F and H), Foxo is distributed evenly throughout the cell body. When Hep^{act} is expressed (G and I), Foxo concentrates in the nucleus. Arrowheads are added as orientation.

(J and K) IPC-specific JNK activation represses growth of the organism in a DFoxo-dependent manner. Dilp2-Gal4 was used to activate JNK in IPCs by overexpression of Hep^{act}. Size differences are visible in sibling flies of different genotypes derived from the same culture (J) and can be quantified measuring the average weight per individual (K). The combined weight of 15 1-day-old individuals was measured. Average weight and standard deviation from three independent measurements are shown. $p < 0.005$ for weight differences between any two genotypes. Genotypes: dilp2G4/UASHep^{act}; +/TM3 |dilp2G4/UASHep^{act}; +/*dfoxo*^{25}| UASHep^{act}/CyO; +/*dfoxo*^{25}.

(L and M) Overexpression of DFoxo in IPCs results in decreased adult size. Size (L) and weight (M) of animals overexpressing DFoxo in IPCs are reduced compared to sibling controls. Genotypes: dilp2G4/UASFoxo | dilp2G4/CyO.

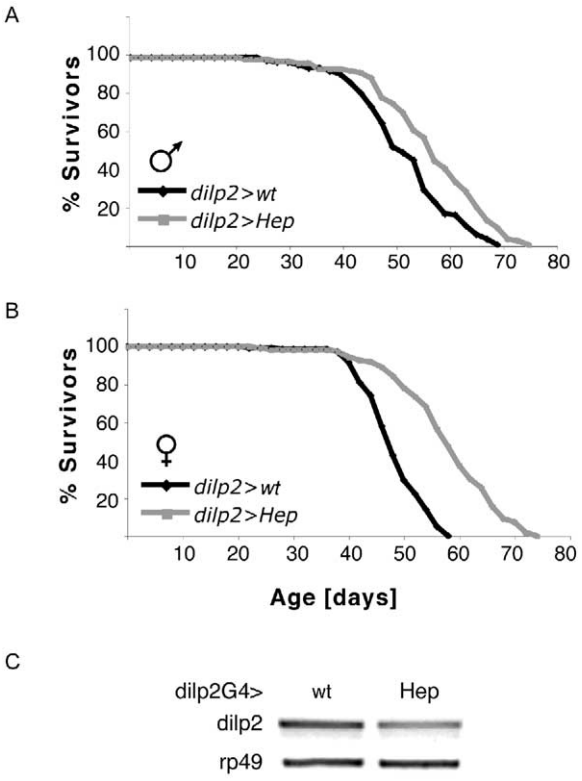

Figure 6. IPC-Specific Increase of JNK Activity Is Sufficient to Extend Life Span

Survival of fly populations at 25°C as recorded from individual cohorts of about 40 flies. Fly stocks were backcrossed into the w^{1118} background. Survival data from separate experiments are combined for clarity. Genotypes and resulting population sizes are as follows:

(A) Males: *w*/Y; dilp2G4/+ n = 126. *w*/Y; dilp2G4/UASHep n = 98.

(B) Females: *w*/+; dilp2G4/+ n = 143. *w*/+; dilp2G4/UASHep n = 110. Statistical analysis was performed using the log rank test. In all cases, $p < 0.001$.

(C) RT-PCR analysis confirms that *dilp2* mRNA levels are moderately decreased in flies overexpressing Hep in IPCs. Results from 3-day-old male flies are shown here.

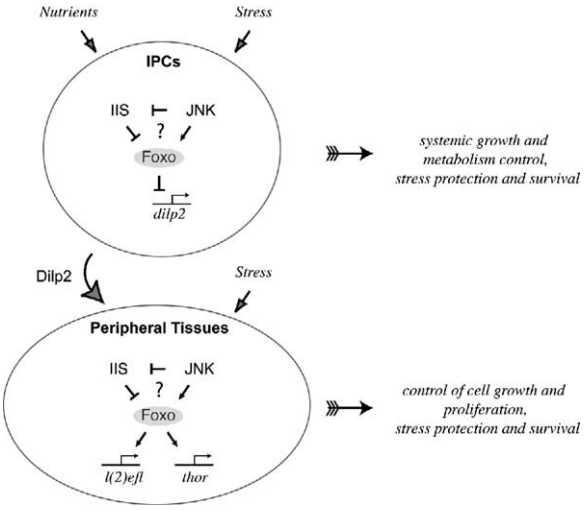

Figure 7. Model for the Coordination of Stress Responses with Growth Control in *Drosophila*

In peripheral tissues, oxidative stress causes cell-autonomous activation of JNK signaling. JNK then suppresses IIS and/or activates Foxo by mechanisms that remain to be elucidated. These signaling events result in the activation of Foxo target genes such as *l(2)efl* and *thor*, which mediate localized stress responses and growth arrest, respectively. JNK/Foxo signaling also promotes systemic changes in growth and metabolism by acting in IPCs, where insulin-like peptides are produced that control IIS activity in peripheral target tissues (Rulifson et al., 2002). JNK activation in IPCs results in a Foxo-dependent decrease of *dilp2* expression. By cell-autonomous as well as systemic mechanisms, IIS and JNK signals thus converge on DFoxo to regulate the transcription of genes involved in growth control, stress protection, and longevity.

(Rulifson et al., 2002), IPCs may thus act as a coordination point for the organism's stress response by down-regulating Dilp production in response to oxidative stress and JNK activation. In target tissues, such a mechanism would induce protective gene expression by the second, cell-autonomous tier of Foxo signaling (Figure 7). Interestingly, the effects of IPC-specific JNK activation on longevity and growth are separable. Life span can be extended by moderately increased JNK activity in IPCs when growth effects are yet not evident. This finding is consistent with observations by others (Clancy et al., 2001; Tu et al., 2002) who showed that the extension of life span in IIS loss-of-function situations is not a mere consequence of small body size.

How did such a multilayered regulation of IIS activity by JNK evolve? It is tempting to speculate that localized activation of Foxo is required to prevent cellular damage and ultimately senescence in conditions in which stressful insults are confined to specific tissues. Such localized insults could, for example, be inflicted by reactive oxygen species that are produced in the environment of amyloid deposits in Alzheimer's disease (Mattson, 2004) as well as by mechanical and oxidative stress experienced by particularly active tissues such as the heart (Wessells et al., 2004). Systemic regulation of Foxo activity, on the other hand, is expected to be an important response mechanism to coordinate metabolism and stress defenses throughout the organism upon changes in the environment. A good example for such a mechanism is the induction of diapause in invertebrates in response to environmental stress or food deprivation (Tatar and Yin, 2001). Accordingly, sensory neurons expressing the insulin-like peptide DAF-28 are required for the induction of the dauer larval stage in response to environmental cues in *C. elegans* (Li et al., 2003).

Systemic and tissue-autonomous effects of JNK/Foxo signaling may be connected in multiple ways. Our data indicate that JNK and Foxo interact in IPCs to repress *dilp2* expression, ultimately activating Foxo in Dilp2 target tissues in a coordinated fashion. Since we found JNK to be activated in IPCs even under normal culture conditions, it is likely that this systemic control of IIS activity by JNK and Foxo plays a critical role in life span regulation. It is, however, also possible that the cell-autonomous protective function of JNK/Foxo signaling is most critical for the survival of specific tis-

sues as the organism ages, thus extending life of the organism by preventing the loss of indispensable cells or tissues. In addition, stress and the JNK-mediated activation of Foxo in peripheral tissues may signal back to IPCs to initiate a systemic response. In *Drosophila*, such a mechanism has been documented in the case of the fatbody. Activation of Foxo in this tissue relays a signal to the IPCs, causing them to curb Dilp2 production, a process that has been proposed to require Foxo activity (Hwangbo et al., 2004). The exact nature of this feedback signaling mechanism in flies is unclear, but it is reminiscent of the complex signaling interactions between β cells and insulin target tissues in mammals (Saltiel and Kahn, 2001). Further studies are required to shed light on the relative contributions of JNK/Foxo signaling in IPCs or Dilp target tissues to life span regulation.

JNK-mediated modulation of IIS activity is likely to be evolutionarily conserved. Inhibitory crosstalk from JNK to IIS in mammalian cells has been found to occur by JNK-mediated phosphorylation and inhibition of IRS-1 (Hirosumi et al., 2002; Lee et al., 2003). This interaction is responsible for obesity-induced insulin resistance in mice (Hirosumi et al., 2002). Whether mammalian homologs of Foxo take part in this pathology remains to be determined. A second possible mechanism for JNK/IIS pathway interaction is the direct phosphorylation and activation of Foxo by JNK. A recent study supports such a mechanism, showing that in mouse cells JNK can phosphorylate the DFoxo homolog Foxo4 in response to oxidative stress (Essers et al., 2004). The physiological relevance of this phosphorylation event has not yet been addressed. The JNK target residues on IRS-1 and Foxo4 are not conserved in the *Drosophila* homologs Chico and DFoxo, and further studies are thus required to determine whether JNK-Foxo crosstalk in *Drosophila* is mediated via homologous mechanisms.

The systemic regulation of IIS activity by JNK and Foxo appears to be conserved as well. It has been suggested that *C. elegans* Daf16/Foxo regulates life span (at least in part) by reducing the expression of insulin-like peptides (Libina et al., 2003; Murphy et al., 2003). In mammals, pancreatic β cells (the counterparts of IPCs) reduce their production of insulin in response to oxidative stress-mediated JNK activation (Kaneto et al., 2002). Conversely, dephosphorylation of JNK by MAPK phosphatase 1 can induce insulin expression in these cells (Zhang et al., 2003). Reducing circulating insulin levels by JNK-mediated Foxo activation may thus be a general mechanism that balances growth and metabolism with stress defense and damage repair.

Experimental Procedures

Drosophila Strains and Antibodies

Fly strains were the following: *elav*^{C155}Gal4 from the Bloomington *Drosophila* stock center; *puc*^{E69}, *ry*^{506}/TM3, gift from E. Martín-Blanco; *arm*Gal4, UASHep, UASHep^{act}, *GMR*Gal4, *sep*Gal4, and UASBsk^{DN}, gifts from M. Mlodzik; UASInR, *Dfoxo*^{21}/TM3, and *Dfoxo*^{25}/TM3, gifts from E. Hafen; UASDfoxo, gift from O. Puig; and *dilp2*Gal4, gift from E.J. Rulifson.

UAS l(2)efl transgenic flies (*w*^{1118} background) were generated by P element mediated transformation in our laboratory. cDNA purified from adult flies was used to amplify the full-length coding sequence of the *l(2)efl* gene (as annotated in FlyBase) via PCR. The resulting fragment was sequenced and cloned into the EcoRI and XbaI sites of pUAST.

Antibody against Dfoxo was a gift from O. Puig (Puig et al., 2003). Antibody against Dilp2 was a gift from E.J. Rulifson (Rulifson et al., 2002). Rat anti-ELAV was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

Life Span Measurements

Survival curves were recorded as described (Wang et al., 2003). Cohorts of about 100 males or females were separated 2 days after hatching and transferred into fresh vials at defined densities (100 flies per 50 ml food). Flies were transferred into fresh vials every 4 days (every 2 days for experiments in Figure 6), and the number of dead flies was recorded. Progeny from crosses of *puc*^{E69}, *ry*^{506}/*TM3* females or coisogenic *ry*^{506}/*TM3* females with *w*^{1118};;*Dfoxo*^{21}/*TM3* males, *w*^{1118};;*dfoxo*^{25}/*TM3* males, or *w*^{1118} males was used to determine life span in Figure 1.

Clonal Analysis in Fatbody

Clones of cells expressing Gal4 were generated in the fatbody using the Flp-out Gal4 technique (Britton et al., 2002). Flies carrying hsFlp; Act>CD2>Gal4, UASeGFP were crossed with flies carrying UAS-linked transgenes. At late second instar larval stage, the progeny was heat shocked once at 37°C for 2 hr to induce Flp expression. Fatbody tissue from third instar larvae was stained with anti-DFoxo antibody (1:200). GFP fluorescence served as cell marker for transgene expression.

Immunostaining and X-Gal Staining

Brains of adult flies were dissected in PBS and fixed in 4% formaldehyde in PBS overnight at 4°C. Brains were stained using anti-Dilp2 antibody (1:200), anti-β-galactosidase antibody (ICN, 1:1000), and fluorescent secondary antibodies. Fatbody tissue was dissected in PBS/0.1% Triton X-100 from third instar larvae and fixed in 4% paraformaldehyde/PBS/0.1% Triton X-100 at room temperature. Images were taken on a Leica SP2 confocal microscope. For X-Gal staining, brains were dissected and fixed in 4% paraformaldehyde/PBS. Staining was carried out at 37°C in staining buffer containing 10 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2$, 10 mM $K_4(Fe[CN]_6)$, 10 mM $K_3(Fe[CN]_6)$, and 0.1% Triton X-100.

RT-PCR

Total RNA was isolated using TRIzol (GIBCO-BRL), and RT-PCR was performed as previously described (Jasper et al., 2001; Wang et al., 2003). Primers included: *Thor* (sense, 5′-CACTTGCGGAAGG GAGTACG-3′, and antisense, 5′-TAGCGAACAGCCAACGGTG-3′), *l(2)efl* (sense, 5′-AGGGACGATGTGACCGTGTC-3′, and antisense, 5′-CGAAGCAGACGCGTTTATCC-3′), *dilp2* (sense, 5′-TCCACAGTG AAGTTGGCCC-3′, and antisense, 5′-AGATAATCGCGTCGACCAGG-3′), *puc* (sense, 5′-CGAGGATGGGTTTGATTACGA-3′, and antisense, 5′-TCAGTCCCTCGTCAAATTGCT-3′), and *rp49* (sense, 5′-TCCTACCAGCTTCAAGATGAC-3′, and antisense, 5′-CACGTTGTG CACCAGGAACT-3′).

Acknowledgments

We thank H. Land and G. Sykiotis for critical reading of the manuscript and E. Hafen, E. Rulifson, M. Mlodzik, E. Martin-Blanco, O. Puig, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank at the University of Iowa for reagents and fly lines. O. Puig is acknowledged for helpful discussions and communication of unpublished results. This work was supported by the Nathan Shock Center of Excellence in the Basic Biology of Aging at the University of Rochester and by a grant from the Upstate Coalition for Aging Research—American Federation for Aging Research to H.J.

Received: November 8, 2004
Revised: January 20, 2005
Accepted: February 28, 2005
Published online: March 24, 2005

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Note Added in Proof

A function for JNK-1 in regulating Foxo/DAF-16 activity has now been found in *C. elegans*. This study demonstrates that the role of JNK signaling in the regulation of life span and stress tolerance is evolutionarily conserved. It confirms our results and interpretations presented here and is published as the following: Oh, S.W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R.J., and Tissenbaum, H.A. (2005). JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc. Natl. Acad. Sci. USA *102*, 4494–4499.