Developmental Cell, Vol. 5, 811–816, November, 2003, Copyright ©2003 by Cell Press

JNK Signaling Confers Tolerance to Oxidative Stress and Extends Lifespan in *Drosophila*

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Summary

Changes in the genetic makeup of an organism can extend lifespan significantly if they promote tolerance to environmental insults and thus prevent the general deterioration of cellular function that is associated with aging. Here, we introduce the Jun N-terminal kinase (JNK) signaling pathway as a genetic determinant of aging in *Drosophila melanogaster*. Based on expression profiling experiments, we demonstrate that JNK functions at the center of a signal transduction network that coordinates the induction of protective genes in response to oxidative challenge. JNK signaling activity thus alleviates the toxic effects of reactive oxygen species (ROS). In addition, we show that flies with mutations that augment JNK signaling accumulate less oxidative damage and live dramatically longer than wild-type flies. Our work thus identifies the evolutionarily conserved JNK signaling pathway as a major genetic factor in the control of longevity.

Introduction

Reactive oxygen species (ROS) damage biological macromolecules (lipids, nucleic acids, and proteins) by oxidizing them and altering their structure and function. Oxidative damage to DNA and proteins has been implicated in a variety of degenerative diseases (among them Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and rheumatoid arthritis; Stadtman, 2001). In addition, accumulation of genomic defects caused by ROS throughout the lifetime of an organism may result in increased cancer risk (Beckman and Ames, 1998; Feig et al., 1994; Grollman and Moriya, 1993). According to the "free radical theory of aging" (Beckman and Ames, 1998; Harman, 1956), the collective damage wrought by ROS is also believed to be a major cause for the general deterioration of cell and tissue function with progressing age (Tissenbaum and Guarente, 2002). To counteract these injuries, aerobic organisms employ a variety of molecules and mechanisms that prevent or relieve damage inflicted by ROS, some of which have been shown to extend lifespan (Beckman and Ames, 1998; Finkel and Holbrook, 2000). It is as yet not fully elucidated how the activity of this protective system is regulated following oxidative insults. Stress-sensing signaling pathways include several MAP kinase cascades involving the "stress-activated protein kinases" (SAPKs) p38 and JNK (Davis, 2000; Paul et al., 1997; Stronach and Perrimon, 1999). The JNK signaling cascade is triggered by a variety of insults, including UV radiation and oxidative stress. The consequences of this activation in vivo are not well understood. At the cellular level, it may serve a protective function (Minamino et al., 1999) but may also promote apoptosis (Tournier et al., 2000). Here, we analyze the role of JNK, and its downstream genetic program, in oxidative stress tolerance and aging of the organism.

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Results and Discussion

JNK Induces a Gene-Expression Program of Oxidative Stress Response in the Organism

JNK phosphorylates a variety of transcription factors and enhances their transcriptional activation potential. Thus, insight into the biological consequences of stress-activated JNK signaling might be gained by analyzing the relevant downstream genetic programs. We chose *Drosophila* as a model organism for such studies, as its JNK pathway is genetically very tractable. The multiplicity of homologous and functionally at least partially redundant kinases (three mammalian JNK genes produce at least ten protein isoforms; Davis, 2000) has impeded similar analyses in mammals. We recently mapped the genomic response to JNK signaling in the *Drosophila* embryo using serial analysis of gene expression (SAGE; Jasper et al., 2001). Among the genes induced in embryos with increased JNK signaling, we identified a group with tentative functions in cellular stress responses as well as several genes that were known to be activated in response to oxidative damage (Figure 1, and Supplemental Table S1 available online at http://www.developmentalcell.com/cgi/content/full/5/5/811/DC1; Jasper et al., 2001). In an independent experiment, we found similar genes upregulated in response to JNK signaling in differentiating photoreceptors (H.J., unpublished data and Supplemental Table S2). These findings suggested that JNK signaling activates a gene expression program that confers tolerance to oxidative stress in a variety of cell types. To test this hypothesis, we monitored the expression of four representative genes (*hsp68*, *gstD1*, *fer1HCH*, and *mtnA*), which were identified as JNK dependent in our SAGE experiments, in the adult fly using quantitative real-time RT-PCR. The induction of the respective mRNAs in response to oxidative stress, artificially brought on by treatment with the drug paraquat, was measured in wild-type flies and in hemizygotes for hep^1, a hypomorphic allele of the *Drosophila* JNKK gene, *hemipterous* (*hep*; Glise et al., 1995). Paraquat, a compound widely used to apply oxidative stress to cells and organisms, leads to continuous intracellular generation of $O_2^{\cdot-}$ radicals (Arking et al., 1991). It efficiently activates JNK in the fly, as indicated by the transcriptional activation of *puckered* (*puc*), one of the prototypical target genes of JNK signaling in *Drosophila* (Figure 1). *puc* encodes a JNK-specific phosphatase that downregulates the pathway, thus establishing a negative feedback loop (Martin-Blanco et al., 1998). RT-PCR data show that JNK signal-

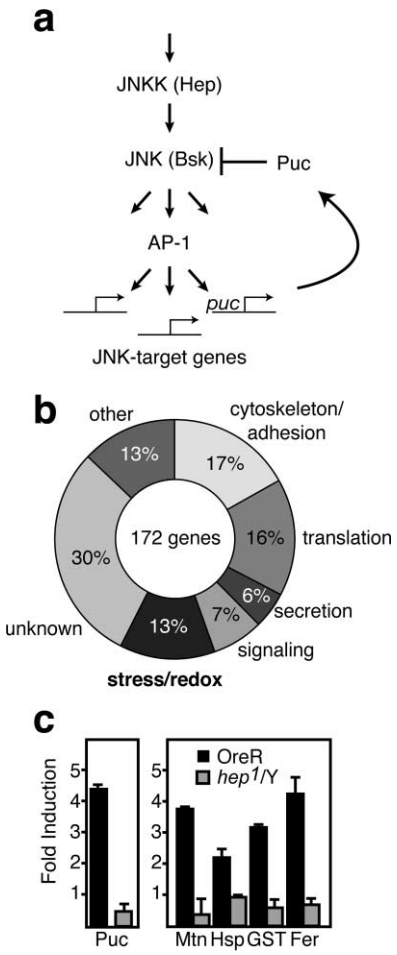

Figure 1. Oxidative Stress Response Genes Regulated by JNK Signaling In Vivo

(A) JNK signal transduction in *Drosophila*. JNKK and JNK are encoded by *hemipterous* (*hep*) and *basket* (*bsk*), respectively. The AP-1 transcription factor complex, consisting of Jun and Fos, is activated by phosphorylation. Puckered, a VH1 phosphatase of Bsk, is induced by AP-1 and downregulates the pathway.

(B) Functional breakdown of genes induced after JNK activation in the *Drosophila* embryo (SAGE experiment described in Jasper et al., 2001). A prominent group includes genes involved in redox regulation and stress responses. See Supplemental Table S1 for details.

(C) Requirement of JNK signaling for the induction of selected stress-response genes by oxidative stress. Quantitative RT-PCR using mRNA from 1-day-old adult males (wild-type, OreR; JNK-signaling deficient, *hep*^{1} hemizygotes) fed with paraquat (20 mM in 5% sucrose) for 90 min (60 min for *puc*). RNA quantities are normalized to rp49 transcript levels and to samples from mock-treated flies. Means and standard deviations from three independent experiments are shown. Activation of *puc* is indicative of JNK signaling activity. Metallothionein A (MtnA) is induced by a variety of environmental stresses and is believed to play an important antioxidant role (Nath et al., 2000). Heat shock protein 68 (Hsp68) is closely related to Hsp70. Its induction after oxidative stress (Gosslau et al., 2001) may relieve protein damage by ROS. Glutathione S Transferase D1 (GSTD1) is induced in aging and paraquat-exposed flies (Zou et al., 2000). Ferritin 1 heavy chain (Fer1HCH) scavenges free iron, a major generator of ROS through the Fenton reaction. Induction of *ferritin* after oxidative stress as well as its regulation by AP-1 has been described in the mouse (Tsuji et al., 1998). The lack of induction of these genes in *hep*^{1} hemizygotes indicates a requirement for JNK signaling in the oxidative-stress response in *Drosophila*.

ing is required for the induction of the four tested genes in response to oxidative stress (Figure 1), supporting the notion that flies react to oxidative challenge with a protective gene expression program dependent, at least in part, on JNK signal transduction.

Oxidative Stress Tolerance Induced by JNK Signaling in *Drosophila*

To examine the relevance of JNK signaling for the sensitivity of the organism to oxidative stress, we exposed adult flies to paraquat for a prolonged period of time and monitored their survival (Figure 2). Compared to wild-type animals, flies with decreased JNK signaling potential (hemizygotes for *hep*^{1}, or heterozygotes for a hypomorphic allele of the *Drosophila* JNK gene *basket, bsk*^{2}) were more sensitive to moderate doses of paraquat. Conversely, flies gained resistance to paraquat when signal flow through the kinase cascade was promoted by overexpression of Bsk or Hep. Similarly, boosting JNK signal transduction by reducing the gene dose of *puc*, conferred strong paraquat resistance in a *hep*- and *bsk*-dependent fashion (Figure 2). Flies heterozygous for *puc* exhibit elevated levels of JNK activity, as inferred by the dosage sensitivity of JNK-mediated apoptotic phenotypes in the developing wing (Adachi-Yamada et al., 1999), as well as by rescue of developmental defects normally observed in flies carrying *hep* and *kay* mutations (Zeitlinger and Bohmann, 1999). Constitutive overexpression of one of the identified JNK-inducible stress response genes, Hsp68, also protects flies against oxidative stress, suggesting that JNK's downstream genetic program mediates the observed protection (Figure 2). The observed differences in sensitivity to paraquat were not due to feeding abnormalities or a general tolerance to toxic compounds of the tested genotypes, as they are similarly sensitive to G418 toxicity (Figure 2B).

Tissue-specific overexpression of superoxide dismutase (SOD) in motorneurons increases the resistance to oxidative stress and extends the lifespan of *Drosophila* (Parkes et al., 1998). This result suggests neurons as the "weakest link" in the organism's tolerance to oxidative insults and as a cell type in which protective mechanisms would be most critical. To investigate whether JNK signaling in neurons could play a role in such mechanisms, we examined fly strains in which Hep overexpression was directed either to the nervous system or to muscle tissue in an RU486-inducible manner (using the "gene-switch Gal4" driver; Osterwalder et al., 2001). The toxicity of paraquat was reduced significantly when Hep was expressed in the nervous system (ELAV Gal4 drives expression in all cells of the peripheral and central nervous systems; Luo et al., 1994; Yao and White, 1994) but not when it was expressed in the musculature (Figure 2). This result highlights a specific function of JNK signaling in the protection of neurons against oxidative stress. While we cannot rule out that JNK may also act protective in nonneuronal cells (for instance in muscle cells), such protection seems not to be sufficient for the organism's survival, indicating that protection of neurons is critical.

Importantly, the inducibility of the JNK effect by RU486 in this system rules out variations in the genetic back-

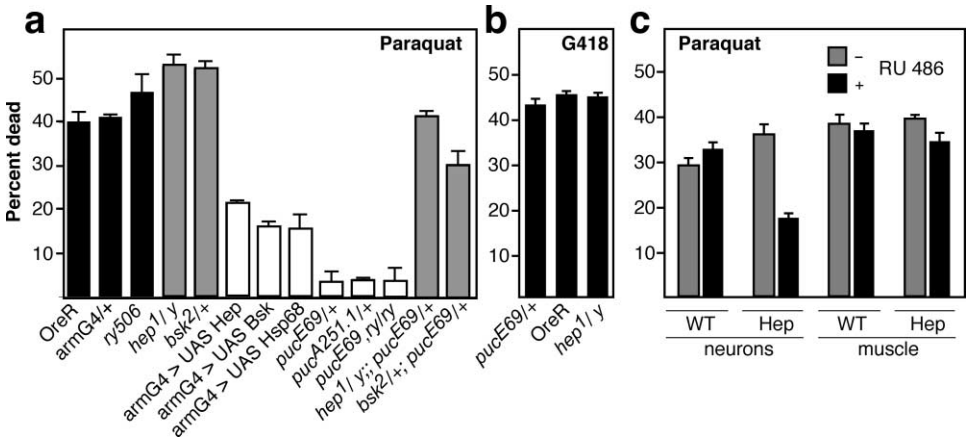

Figure 2. JNK Signaling Confers Tolerance to Oxidative Stress in *Drosophila*

(A) Mortality in cohorts of 100 males each after feeding 15 mM paraquat in 5% sucrose for 18 hr. Reduced JNK signaling (hep^1/y and $bsk^2/+$) results in hypersensitivity to paraquat compared to wild-type (OreR and armG4/+), while increase in signal flow (*puc*/+ or ubiquitous overexpression of Bsk or Hep using armGal4) rescues lethality. This rescue is less efficient in flies double heterozygous for *puc* and *hep* or *bsk*. The canonical JNK signaling pathway thus seems to mediate the observed effects. Mean and standard deviation of three or more independent experiments are depicted. Flies carrying *puc* and *hep* mutations were crossed with the OreR strain to minimize differences in genetic backgrounds. Two different *puc* alleles were used to rule out the influence of different genetic backgrounds in the observed effects: puc^{E69} and $puc^{A251.1}$ are alleles generated independently in different laboratories by P element insertion into the second intron of the *puc* gene (Martin-Blanco et al., 1998). In addition, isogenic controls were generated by backcrossing puc^{E69},*ry*/TM3 into the ry^{506} genetic background. Constitutive overexpression of the JNK-inducible gene *hsp68* also protects against paraquat-induced lethality.

(B) Flies fed with G418 (10 mg/ml in 5% sucrose) die at similar rates regardless of their genotype. Death rate was assessed after feeding G418 for 36 hr.

(C) Overexpression of Hep in neurons (Gene Switch Gal4 controlled by *elav* promoter) but not in muscle (GSGal4 controlled by *myosin heavy chain* promoter) increases tolerance to paraquat. Similar effects were observed when overexpressing Bsk (M.C.W. and H.J., unpublished data). Genotypes: +/CyO; elavGSGal4/+ (wt neurons); UASHep/CyO; elavGSGal4/+ (Hep neurons); +/CyO; MHC GSGal4/+ (wt muscle); UASHep/CyO; MHC GSGal4/+ (Hep muscle).

One hundred percent of flies of any genotype survive when fed with five percent sucrose alone (M.C.W. and H.J., unpublished data). Similar data were obtained with female flies (M.C.W. and H.J., unpublished data). Statistical significance was determined using a Student's t test. P values are as follows: OreR/hep^1, $p = 2 \times 10^{-5}$; OreR/bsk^2, p = 0.01; OreR/puc^{E69}, $p = 2 \times 10^{-8}$; *ry*/puc^{E69},*ry*, p = 0.008; puc^{E69}/hep^1;; puc^{E69}, p = 0.002; puc^{E69}/bsk^2;puc^{E69}, p = 0.002; armG4/armG4,UASHep, p = 0.0005; armG4/armG4,UASBsk, p = 0.0003; armG4/armG4,UASHsp68, p = 0.002; Geneswitch experiments: wt neurons, p = 0.01; Hep neurons, p = 0.0027; wt muscle, p = 0.27; Hep muscle, p = 0.024.

ground as an explanation for differences in paraquat sensitivity.

Lifespan Extension by Elevated Levels of JNK Signaling

According to the free radical theory of aging (Harman, 1956), one genetic determinant for the lifespan of an organism is its sensitivity to oxidative stress. We asked whether the protection against oxidative damage that is brought about by an increase in JNK signaling potential might be sufficient to extend *Drosophila*'s life expectancy. We examined flies heterozygous for *puc* to test this hypothesis, since our experiments demonstrated that the tolerance of flies to oxidative stress increases with decreasing gene dose of *puc*. Flies heterozygous for either one of two different loss-of-function alleles of *puc* ($puc^{A251.1}$ or puc^{E69}; Martin-Blanco et al., 1998) showed dramatic extensions of median and maximum life expectancy compared to wild-type flies and to flies of an isogenic control strain (Figures 3A, 3C, and 3D). The difference in the degree of lifespan extension by the two alleles correlates well with their described allelic strength (McEwen et al., 2000). Our results thus suggest a direct relationship between the decrease of Puc activity in the mutants and the resulting lifespan extension. Since biochemical and genetic data indicate that the activity of Puc is limited to the JNK signaling pathway (as opposed to other MAPK pathways; Martin-Blanco et al., 1998), the lifespan extension in *puc* mutants is likely to be caused by higher levels of JNK signaling. We tested the requirement for a functional JNK pathway in the longevity of *puc* mutants directly by comparing the lifespan of puc^{E69} heterozygous males in a wild-type background to puc^{E69} heterozygotes in a hep^1 hemizygous backgound (Figure 3B). Heterozygosity for *puc* leads to an only modest increase in mean and maximum lifespan of hep^1 hemizygous flies, indicating that a functional JNK cascade is required for efficient lifespan extension in *puc* mutants. These results strongly support the notion that the longevity phenotype observed in *puc* mutants is due to an increase in JNK signaling activity.

Our genomic experiments suggested that elevated JNK signaling activity causes higher basal levels of protective genes. We tested whether constitutive overexpression of one of the identified JNK target genes, *hsp68*, would be sufficient to extend lifespan of *Drosophila* (Figure 3E). In agreement with our hypothesis, we observed small but significant increases in mean and maximum lifespan in flies that overexpress *hsp68* compared to isogenic wild-type controls. This experiment is consistent with earlier observations in which increased expression of chaperones extended the lifespan of *Drosophila* (Tatar et al., 1997).

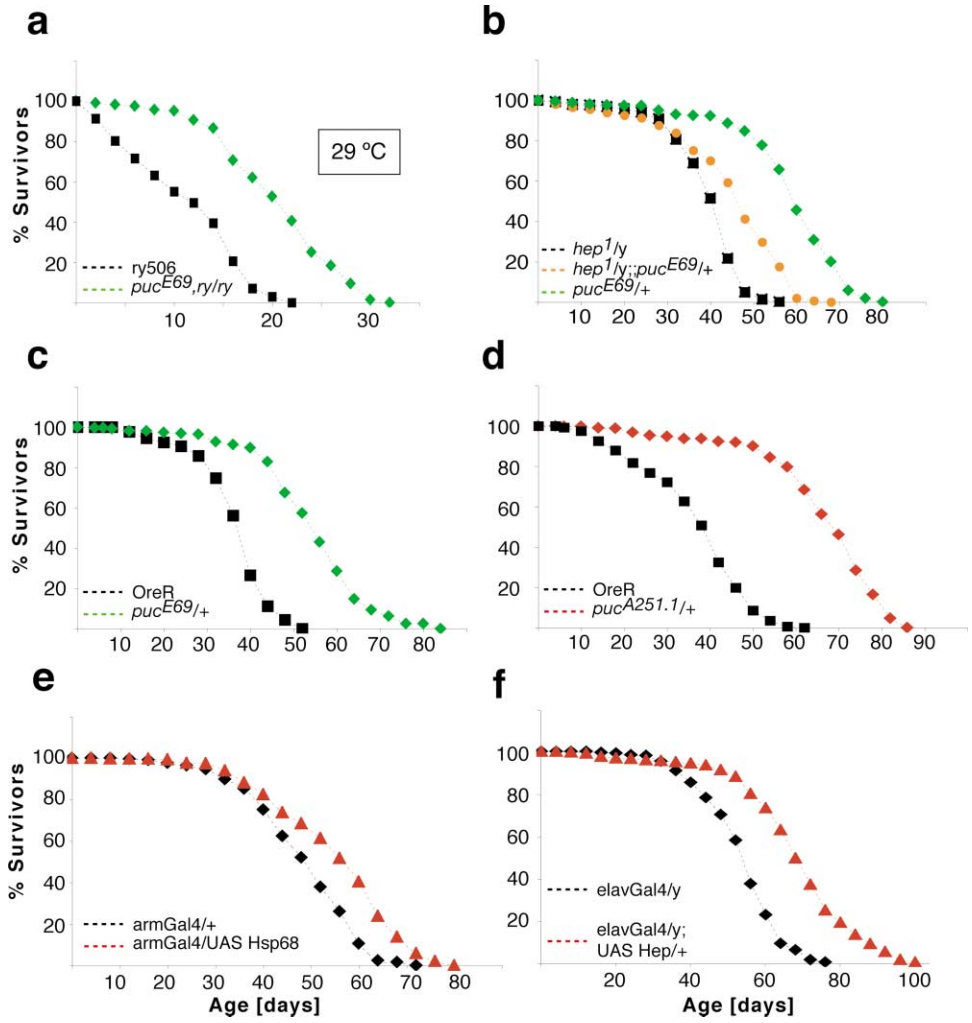

Figure 3. Increased JNK Signaling Potential Extends Lifespan in *Drosophila*

(A) Heterozygotes for puc^{E69} (green diamonds) have a longer life span than flies that are otherwise isogenic (10× backcrossed, black squares). Note that lifespan in these experiments was generally shorter than in the following ones, due to cultivation at 29°C.

(B) The extension of lifespan by *puc* heterozygosity requires functional Hep. hep^1 hemizygous males (black squares) exhibit lifespan comparable to wild-type flies. Reduction of the *puc* gene-dose only marginally extends lifespan in these flies (orange circles). Compared to *puc* mutants in a wild-type background, hep^1 reduces lifespan.

(C and D) Males heterozygous for two different *puc* alleles (progeny from crosses between *puc*/TM3 and OreR) show significant extension of mean and maximal lifespan when compared to wild-type flies. Females show qualitatively similar lifespan data, even though the extension of lifespan in puc^{E69} females is less pronounced than in males (data not shown).

(E) Constitutive overexpression of the JNK target gene *hsp68* extends lifespan. w^{1118};;P{w^{+mC}armGal4} flies were crossed to w^{1118} or w^{1118};P{w^{+mC}UASHsp68}, and survival of the male progeny was monitored at 25°C.

(F) Increased JNK signaling in neurons is sufficient to extend lifespan. w^{1118},P{w$^{+mW.hs}$ =GawB}$elav^{C155}$ homozygous females were crossed to w^{1118} or to w^{1118};P{w^{+mC}UASHep} males, and survival of the male progeny was monitored at 25°C.

In all cases, survival in three or more independent cohorts of about 100 flies each was monitored over time. Here, these data are combined into one graph for clarity. Statistical analysis of the combined survival data was performed using the log rank test. Cohort sizes and p values are as follows. (A) *puc*, n = 232; *ry*, n = 192; $p < 0.001$. (B) *hep*, n = 258; *hep,puc*, n = 267; *puc*, n = 338; $p < 0.001$. (C) *puc*, n = 333; OreR, n = 252; $p < 0.001$. (D) *puc*, n = 268; OreR, n = 325; $p < 0.001$. (E) armGal4, n = 679; armGal4 > Hsp68, n = 663; $p < 0.001$. (F) elavGal4, n = 360; elav > Hep, n = 320; $p < 0.001$.

Unless stated otherwise, flies were reared at 25°C.

Providing higher JNK signaling levels in neuronal tissue is sufficient to increase oxidative stress tolerance (Figure 2C). To test whether neuronal-specific protection would also be sufficient to extend lifespan of the organism, we monitored survival in flies that overexpress Hep constitutively in neuronal tissue under the control of ELAV Gal4. Neuronal overexpression of Hep extended lifespan significantly, indicating that the level of JNK activity in neuronal tissue determines not only the fly's oxidative stress tolerance, but also its lifespan. Importantly, these results confirm, independently of *puc* mutations, that JNK signaling promotes longevity.

Several genetically determined changes in physiology have been associated with extended lifespan in *Drosophila*. Such changes include reduced reproductive activity, dwarfism, delays in development, as well as stress

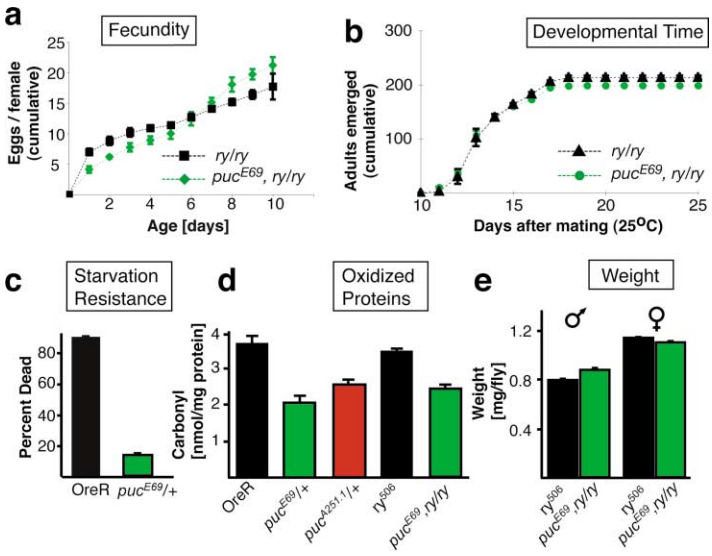

Figure 4. Aging-Relevant Phenotypic Characteristics of *puc* Heterozygotes

(A, B, and E) *puc* mutants show no reduction in fecundity (A), developmental speed (B), and weight (E, average of 50 flies) compared to isogenic controls. Mean and standard deviations from three independent experiments are shown in (A), (B), and (E). (C) *puc* heterozygotes are less sensitive to dry starvation. Lethality after 24 hr of starvation is shown (n = 100; 2-day-old males). Mean and standard deviations from three independent experiments are shown. (D) Ten-day-old *puc* heterozygotes contain less oxidized proteins than wild-type flies or isogenic controls of the same age as measured by a decreased protein carbonyl content. Mean and standard deviations from five independent experiments are shown (Student's t test: $p = 0.0004$ for $puc^{E69} \leftrightarrow$ OreR; $p = 0.0013$ for $puc^{A251.1} \leftrightarrow$ OreR, and $p = 0.0009$ for puc^{E69},*ry*/*ry* ↔ *ry*/*ry*).

tolerance (Clancy et al., 2001; Tu et al., 2002). We examined whether the JNK pathway might affect parameters indicative of such physiological changes (Figure 4). *puc* heterozygotes and wild-type controls exhibit roughly equivalent sizes (as determined by body weight, Figure 4E), reproductive activities (fecundity, Figure 4A), as well as developmental timing (Figure 4B). In contrast, oxidative stress tolerance and tolerance to starvation differ markedly between wild-type and *puc* heterozygous flies (Figures 2 and 4C). Importantly, 10-day-old *puc* heterozygotes contain significantly decreased levels of oxidized proteins (Figure 4D). The quantity of protein oxidation products, such as polypeptides carrying carbonyl groups, is a measure for the accumulated oxidative damage suffered by an organism (Stadtman, 2001). Taken together, our results suggest that increased JNK signaling is sufficient to reduce oxidative damage throughout the lifetime of a fly and that this beneficial effect may be the cause of the longevity phenotype of gain-of-function mutants for this signaling pathway.

This work identifies the JNK signaling pathway as a significant genetic determinant of longevity in *Drosophila*. Activation of JNK in response to oxidative challenge and to other environmental insults has been well described in a number of model systems and was proposed to trigger the expression of genes that could mediate protective functions on the organism at least in certain cell types. Against this backdrop, resulting in the prediction that JNK signaling would protect the organism from oxidative challenge (which we show here to be correct), it may seem surprising that, until now, no evidence has been produced that links JNK signaling to an extended lifespan. Evidently, experimental limitations of mammalian systems, including increased functional complexity and genetic redundancy, have precluded clear-cut experiments to address this question.

While we cannot exclude unidentified functions of JNK signaling that might be relevant to the aging process, it seems plausible (and the free radical theory of aging would predict) that the observed protection against oxidative insults decisively delays aging and thus causes the longevity phenotype of *puc* heterozygotes. Earlier observations, as well as our experiments, support this notion: Hsp70, and its JNK-inducible relative Hsp68, extend lifespan when overexpressed in *Drosophila* (Figure 3G and Tatar et al., 1997). These chaperones have been implicated in oxidative stress resistance (Gosslau et al., 2001) and may have repair functions downstream of JNK signaling. The reduced level of oxidative damage in aging *puc* heterozygotes (Figure 4E) further supports this view. JNK signaling thus emerges as an evolutionarily conserved gene-regulatory network that limits oxidative damage in the organism and its impact on aging.

Experimental Procedures

Genetics and Fly Handling

Fly strains used were OreR, from Bloomington stock center; hep^1/FM6, gift from S. Noselli; puc^{E69},*ry*/TM3, and $puc^{A251.1}$,*ry*/TM3, gifts from E. Martín-Blanco; UASHep and UASBsk, gifts from M. Mlodzik; elav-Gal4, elav-GSGal4, and MHC-GSGal4, gifts from M. Ramaswami. Fly lines carrying UASHsp68 transgenes were generated by P element-mediated transformation of w^{1118} mutant flies. The Hsp68 cDNA was cloned using PCR to amplify the full-length cDNA sequence (as annotated in Flybase) and ligating it into the NotI and KpnI sites of pUAST. Unless stated otherwise, flies were reared at 25°C and 65% humidity on cornmeal- and molasses-based food. All experiments were conducted with flies that developed at equal larval densities.

For paraquat treatments, flies were starved for 6 hr and then transferred to vials containing filter paper soaked in 5% sucrose solution with or without 15 mM paraquat (Methyl Viologen, Sigma). Survival was assessed after 18 hr. Flies were kept in the dark at all times.

RU486-induction was performed by feeding flies with 400 μg/ml RU486 in 5% sucrose on filter paper for 1.5 hr. Filter paper was then exchanged for filter paper soaked with 400 μg/ml RU486 and 20 mM paraquat in 5% sucrose. Survival was assessed after 20 hr. Compared genotypes were always treated in parallel.

To monitor lifespan, cohorts of about 100 males or females were separated after mating for 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. To generate an isogenic control line for *puc* mutants, puc^{E69},*ry*/TM3 flies were backcrossed 10 times into the ry^{506} background using the ry^+ P element inserted in the *puc* locus as a marker. In additional experiments (Figure 3C and 3D), the lifespan of *puc*/+ male progeny from crosses of *puc*/TM3 mutants with OreR wild-type controls was monitored. The reference background for transgenic lines (elavGal4, armGal4, UASHepwt, and UASHSP68) was w^{1118}. Driver lines were crossed

with either w^{1118} or with the corresponding UAS lines and the lifespan of male progeny was assessed.

Fecundity was measured by counting the number of eggs laid each day for the first ten days after mating. Genotypes were compared by counting eggs laid by *puc,ry/ry* females that were mated with *puc,ry/ry* males; or by *ry/ry* females mated with *ry/ry* males. Fifty males and one hundred females were allowed to mate for 3 days, then groups of 15 females were separated into individual vials, and the number of eggs laid was determined every 24 hr.

Developmental time was assessed by recording the cumulative number of adults emerged as a function of time. Thirty-five puc^{E69},ry/ry females were crossed with 20 ry^{506} males for 5 days. Starting at 10 days after mating and for an additional 15 days, the number of adults from each vial was counted daily.

Protein Carbonyl Quantification

Protein carbonyl content was measured as previously described (Levine et al., 1990), using 30 10-day-old male flies per genotype homogenized in 500 μl 5 mM phosphate buffer (pH 7.5).

Real-Time RT-PCR

Total RNA was isolated from ten paraquat-treated male flies using Trizol (GibcoBRL). First-strand cDNA was prepared from 5 μg total RNA using Superscript II Reverse Transcriptase (Invitrogen) and oligo $(dT)_{12-18}$ primer in 50 μl reaction volume. Real-time quantitative PCR was performed on an iCycler thermal cycler (Bio-Rad) following the manufacturer's suggestions using SYBR Green-based detection of PCR products. Melting curves were examined after amplification to confirm single-product measurements. Transcript levels were determined from threshold cycle values based on a standard curve generated from serial cDNA dilutions. All reactions were performed in triplicate. Data presented in Figure 1 are from three independent experiments and are normalized to *rp49* as internal control as well as to transcript levels in mock-treated flies.

Acknowledgments

This work was supported by the Nathan Shock Center of Excellence in the Basic Biology of Aging, pilot project no. P30 AG18254. We thank E. Martín-Blanco, S. Noselli, M. Ramaswami, and M. Mlodzik for reagents. H. Federoff, M. Noble, H. Land, J. Homsy, and J. Mendler are acknowledged for helpful comments on the manuscript.

Received: May 22, 2003
Revised: September 3, 2003
Accepted: September 4, 2003
Published: November 3, 2003

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