

Longevity in mice: is stress resistance a common factor?

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Abstract A positive relationship between stress resistance and longevity has been reported in a multitude of studies in organisms ranging from yeast to mice. Several mouse lines have been discovered or developed that exhibit extended longevity when compared with normal, wild-type mice of the same genetic background. These long-living lines include the Ames dwarf, Snell dwarf, growth hormone receptor knockout (Laron dwarf), IGF-1 receptor heterozygote, Little, α -MUPA knockout, p66^{shc} knockout, FIRKO, mClk-1 heterozygote, thioredoxin transgenic, and most recently the Klotho transgenic mouse. These mice are described in terms of the reported extended lifespans and studies involving resistance to stress. In addition, caloric restriction (CR) and stress resistance are briefly addressed for comparison with genetically altered mice. Although many of the long-living mice have GH/IGF-1/insulin signaling-related alterations and enhanced stress resistance, there are some that exhibit life extension without an obvious link to this hormone pathway. Resistance to oxidative stress is by far the most common system studied in long-living mice, but there is evidence of enhancement of resistance in other systems as well. The differences

in stress resistance between long-living mutant and normal mice result from complex inter-relationships among pathways that appear to coordinate signals of growth and metabolism, and subsequently result in differences in lifespan.

Key words mutant mice · lifespan · oxidative stress · hormesis

Introduction

In the aging research community it is well accepted that a positive relationship exists between stress resistance and longevity. There has been a flurry of effort to examine not only resistance to oxidative insults, but exposure to several other cellular environmental perturbations such as temperature, infectious substances, pH and ionic changes, heavy metals, and tumor invasion. These efforts expand our understanding beyond oxidative stress resistance and have heightened the search for the responsible mechanisms. Although there is a large volume of evidence for stress resistance in multiple species that have contributed to our overall understanding of the topic, this particular discussion will be limited to longevous mice.

Stress resistance includes both cellular and whole animal responses to a variety of oxidative and non-oxidative challenges. The initial concept of a link between oxidative stress resistance and longevity is likely derived from the long-standing

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free radical theory on aging introduced by Harman (1956). Free radicals produced as by-products of normal metabolism can damage cells, which over time results in physiological decline. These free radicals, many of which are oxidative in nature, can adversely affect DNA, proteins, and lipids. Three main endogenous cellular systems exist to cope with the metabolically generated free radicals: enzymatic mechanisms, antioxidants, and metal chelators. In addition, a variety of systems within cells have been discovered that can repair damaged cellular components. Without this protection, maintenance of normal physiological function is compromised. There is considerable evidence to prove that the accumulation of damage that has escaped cellular defense mechanisms ultimately leads to aging. In addition, the disposable soma theory of aging suggests that longer-lived species invest more in the durability of their somatic cells and tissues than short-lived species (Kirkwood 1977). Therefore, genes that regulate cell maintenance and repair including stress resistance mechanisms are major determinants of lifespan (Kirkwood 1977; Lithgow and Kirkwood 1996; Johnson et al. 1996; Finkel and Holbrook 2000).

Based on these aging hypotheses, the antioxidative defense capacities were among the first stress-related candidates to be evaluated in several animals with variable longevity and continue to be the first tested in new examples of extended lifespan. Enzymatic processes such as those driven by catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPX), as well as non-enzymatic mechanisms including glutathione (GSH), ascorbic acid, and metallothionein systems within cells and tissues, have been studied. In vivo and in vitro challenges have been utilized to examine resistance to both oxidative and non-oxidative stressors. Other stress resistance mechanisms have also been studied in some of the mice with extended lifespans.

Several mouse lines have been discovered or developed that exhibit extended longevity compared with normal, wild-type mice of the same genetic background. These long-living mutants include the Ames dwarf, Snell dwarf, growth hormone receptor knockout (Laron dwarf), IGF-1 receptor heterozygote, Little, α -MUPA knock-

out, p66^{shc} knockout, FIRKO, mClk-1 heterozygote, thioredoxin transgenic, and most recently the Klotho transgenic mouse. Each will be described in terms of the reported extended lifespan and studies involving resistance to stress. Finally, caloric restriction (CR) and stress resistance will be addressed following the evidence in genetically altered mice.

Ames dwarf mice

Ames dwarf mice exhibit robust longevity with males and females living 49–68% longer than wild-type siblings respectively (Brown-Borg et al. 1996) and the survival curve data suggest that this increase in longevity is due to a delay in age-related mortality. Ames dwarf mice have a primary pituitary deficiency resulting in the absence of growth hormone (GH), prolactin, and thyrotropin. These deficiencies result from a point mutation in *Prop-1*, a gene that promotes appropriate differentiation of the anterior pituitary cells into somatotrophs, lactotrophs, and thyrotrophs (Sornson et al. 1996; Bartke 1979). A significant body of evidence indicates that oxidative stress is influenced by alterations in the GH/IGF-1/insulin signaling pathway. Several components of the antioxidative defense system have been evaluated in Ames mice and reflect an overall upregulated ability to counter oxidative stress. Catalase activity, protein content, and mRNA levels are significantly elevated in liver, kidney, and heart tissues in Ames compared with wild-type mice across the lifespan (Brown-Borg et al. 1999; Brown-Borg and Rakoczy 2000). Catalase and SOD1 (CuZnSOD) are also elevated in the hypothalamus of Ames mice (Hauck and Bartke 2000). The counterpart of catalase in the mitochondria, GPX, is increased in dwarf mouse kidney and liver tissue as is SOD2 (Brown-Borg and Rakoczy 2000 and unpublished data). Skeletal muscle GPX activity is maintained in dwarf mice at rest and following both acute and chronic exercise, yet it declines significantly with age in wild-type mice (Romanick et al. 2004).

In addition to enzymatic systems that counter free radicals, non-enzymatic antioxidant levels

also differ. For example, ascorbic acid levels are decreased in liver tissues of Ames dwarfs (Brown-Borg et al. 1999) while the GSH and metallothionein levels—both of which are capable of scavenging free radicals—are significantly higher in multiple tissues of dwarf mice at several ages relative to corresponding wild-type mice (Brown-Borg et al. 2001b; Meyer et al. 2003). Preliminary data also demonstrate significant increases in Coenzyme Q9 and Q10 levels in the cerebral cortex, striatum, and cerebellar cortex of dwarf mice (Brown-Borg and Sharma, unpublished data). Coenzyme Q10 is a major component of the oxidative phosphorylation and is known to have antioxidant properties.

The functional significance of enhanced ability to counter oxidative stress is apparent in oxidative challenge experiments. Following the administration of paraquat, a systemic oxidative stressor, Bartke and colleagues (2001) showed that dwarf mice survived significantly longer than their wild-type counterparts. Cells isolated from Ames mice also retain stress resistance in culture. Dermal fibroblasts from dwarf mice are markedly more resistant to cell death than fibroblasts from normal mice following treatment with hydrogen peroxide (H_2O_2), UV irradiation, and heavy metal exposure (cadmium; Salmon et al. 2005). In vitro resistance to paraquat toxicity was greater in fibroblasts from dwarf mice, but did not reach statistical significance compared with resistance in wild-type cells.

These enhanced defense abilities are coupled with reductions in oxidative damage to tissue. Inorganic peroxide levels are lower in liver and kidney tissues of Ames mice (Bartke et al. 1998; Carlson et al. 1999). Protein oxidative damage measured as carbonyl formation is nearly 50% lower in liver tissues from old dwarf mice compared with old wild-type mice (Brown-Borg et al. 2001a) and does not rise with age as observed in normal animals. Brain protein carbonyl levels are also lower in long-living dwarf mice. Evidence of differences in oxidative DNA damage has been reported (Brown-Borg et al. 2001a; Sanz et al. 2002). Sanz and co-workers found lower levels of oxidative mitochondrial DNA damage in heart and brain tissues of dwarf mice compared with wild-type mice. Recent data

suggest that Ames mice also accumulate fewer somatic DNA mutations associated with oxidative stress (Jan Vijg, personal communication). Therefore, the observed upregulation in antioxidant defenses in Ames dwarf mice appears to counter reactive oxygen species (ROS) vigorously and reduce overall damage compared with normal animals, resulting in or contributing to extended longevity.

It is worthy of mention that GH administration to dwarf mice, in vivo and in vitro, downregulates the oxidative defense capacity (GPX, catalase, SOD2 [MnSOD]) of these mice (Brown-Borg et al. 2002, Brown-Borg and Rakoczy 2003). In strong support of this work are reports indicating that GH overexpression in vivo suppresses catalase and SOD1, increases tissue oxidative damage, and significantly shortens lifespan (Brown-Borg and Rakoczy 2000; Brown-Borg et al. 1999, 2001b, 2002; Hauck and Bartke 2001; Pendergrass et al. 1993; Wolf et al. 1993; Rollo et al. 1996). Furthermore, IGF-1 appears to regulate antioxidant defenses as the activities of catalase and SOD are suppressed in mouse hepatocytes exposed to GH or IGF-1 (Brown-Borg et al. 2002).

Indications of enhanced stress resistance are found in systems other than antioxidative defense as well. Metallothionein was mentioned as an antioxidant previously. This compound is also

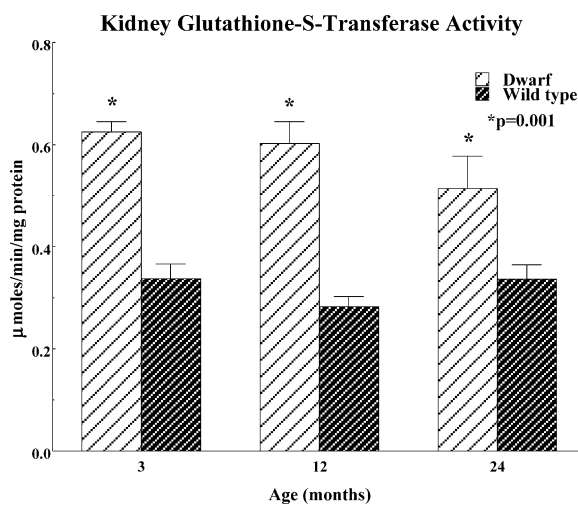


Figure 1 Glutathione-S-transferase activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in kidneys of 3-, 12-, and 24-month-old Ames dwarf and wild-type mice ($n = 6$ mice/genotype/age). Values represent means \pm SEM. $*p = 0.001$

well known as a chelator to counter heavy metal toxicity (cadmium, zinc). Levels of metallothionein are significantly elevated in liver, heart, kidney, and brain striatum of dwarf mice (measured immunocytochemically in tissues and by ELISA; Meyer et al. 2003; Swinscoe et al. 2006a, and unpublished data) relative to levels in age-matched normal animals. In addition, the distribution of various multimeric forms (tetramers, etc.) of metallothionein is different among dwarf, normal and GH transgenic mice (Swinscoe et al. 2006a, 2006b), the relevance of which is currently being evaluated.

Detoxification of endogenous and exogenous compounds are handled by glutathione-S-transferase (GST) enzyme activities. GST activity is significantly increased in dwarf kidney compared with age-matched wild-type mice, certainly contributing to overall stress resistance (Figure 1). In fact, protein expression and activities of multiple players in the GSH pathway are different in the Ames mouse (Brown-Borg and Rakoczy 2005) including reduced levels of γ -glutamyl transpepti-

dase (GGT), the enzyme responsible for GSH degradation in dwarf tissues. The administration of growth hormone opposes the beneficial effects of this pathway by downregulating GST and upregulating GGT activities (Brown-Borg et al. 2005). The overall upregulation in the GSH and the methionine (contributes the cysteine residues for GSH biosynthesis) metabolic pathways (Figure 2) is positively associated with the extended lifespan observed in these mice (Uthus and Brown-Borg 2003). Furthermore, heat shock protein expression is elevated in Ames mice, again suggesting an upregulation of stress defense mechanisms (evident in the kidney; Figure 3). Additional support for enhanced resistance includes evidence that Ames dwarf mice exhibit significantly delayed occurrence of fatal neoplastic disease, reduced severity of both neoplastic and non-neoplastic lesions, and overall reduced disease burden compared with wild-type littermates (Ikeno et al. 2003).

Gene expression analysis also provides evidence of potential enhanced stress resistance in

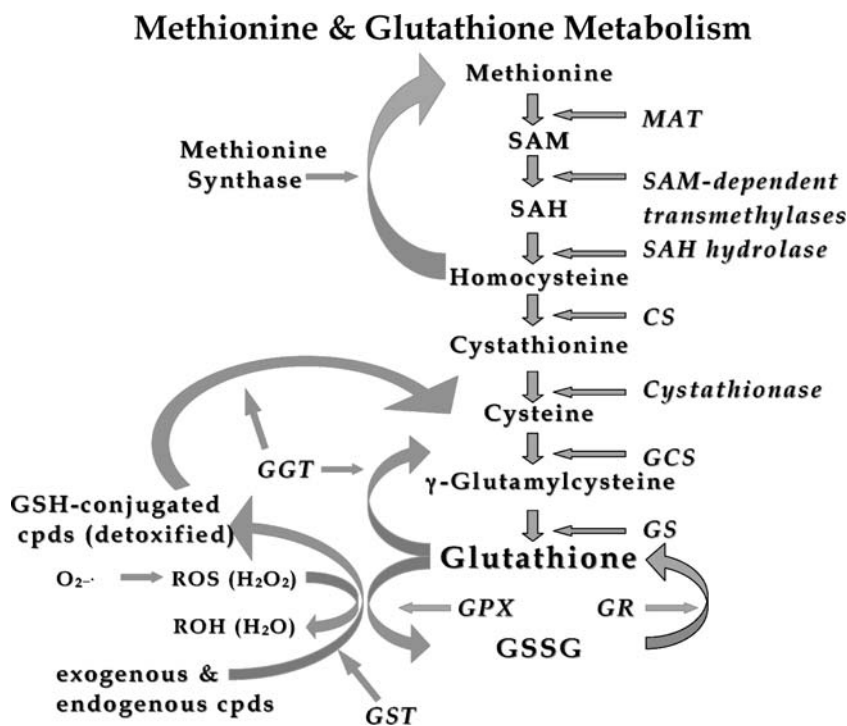


Figure 2 Methionine and glutathione metabolic pathways. *MAT* S-adenosyl methionine synthase, *SAM* S-adenosylmethionine, *SAH* S-adenosylhomocysteine, *CS* cystathionine synthase, *GCS* γ -glutamylcysteine synthe-

tase, *GS* glutathione synthetase, *GGT* γ -glutamyl transpeptidase, *GPX* glutathione peroxidase, *GR* glutathione reductase, *GST* glutathione-S-transferase, *GSSG* glutathione disulfide

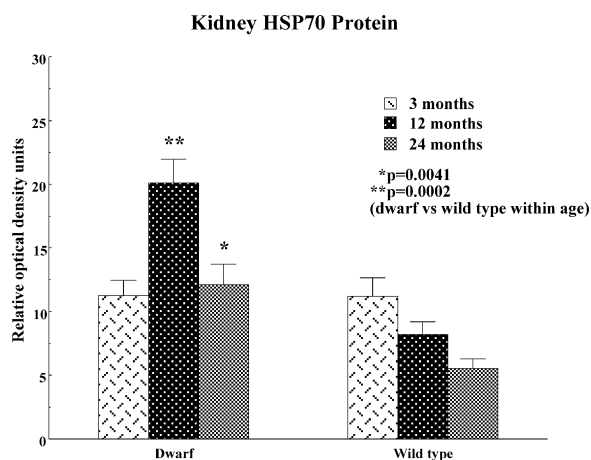


Figure 3 Heat shock protein 70 protein levels in kidney tissues from Ames dwarf and wild-type mice at 3, 12, and 24 months of age. Values represent mean relative optical density units \pm SEM. Comparisons are between genotype within each age. * $p = 0.0041$; ** $p = 0.0002$

Ames dwarf mice. Hepatic expression of mRNA for several GST genes is significantly elevated in dwarf mice over those of wild-type mice (GSTm1, GSTm3, GSTp2, GSTa2, GSTa4; Tsuchiya et al. 2004). Other relevant elevated message levels observed in dwarf mice included GPX4, Gclc (rate-limiting enzyme in GSH metabolism), and several genes involved in both phase I and II xenobiotic metabolism. These findings support evidence that the Ames dwarf mouse exhibits an enhanced repertoire of mechanisms to counter genotoxic/metabolic insults.

At present, only one potential protein repair system has been evaluated in dwarf mice. The methionine sulfoxide reductase (Msr) enzymes function by reducing oxidized methionine (MetO) residues, acting as an antioxidant, and restoring function to damaged proteins. Msr proteins are highly expressed in liver and kidney tissues, suggesting their overall importance in detoxification and therefore maintenance of cellular resistance to oxidative protein damage (Moskovitz et al. 2001; Moskovitz 2005). The levels of MsrB (Selenoprotein B) protein are increased 200% and 115% respectively in liver and kidney tissues of 12-month-old dwarf mice compared with their normal counterparts (Figure 4). This indicates that dwarf mice may have an increased capacity to repair this particular type of protein damage. In contrast, the levels of MsrA protein are actually

somewhat lower in dwarf tissues (54% and 23% for liver and kidney respectively; Figure 4). Preliminary studies indicate that the combined activities of MsrA and MsrB (measured as total Msr activity) of dwarf liver are not different from the wild-type animals, perhaps reflecting the varying protein levels. Further investigation of this protein repair system is underway.

Overall, it appears that many defense or protective systems are upregulated in the Ames dwarf mouse. Multiple components play a role in stress resistance, including antioxidative defense, heat

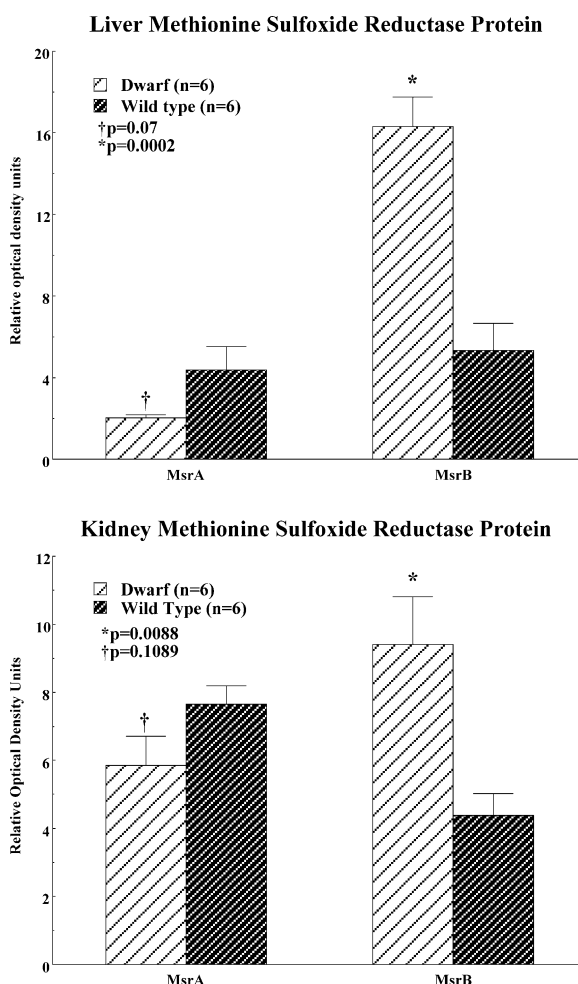


Figure 4 Methionine sulfoxide reductase protein levels in liver and kidney tissues of 12-month-old Ames dwarf and wild-type mice. Antibodies to MsrA and MsrB proteins were utilized to detect relative differences in protein. Values represent mean relative optical density units \pm SEM. Statistical significance for each tissue is indicated

shock proteins, detoxification factors, and glutathione. Each likely contributes to life extension.

Interestingly, the significant extension of lifespan in Ames mice is further increased when caloric intake is restricted (Bartke et al. 2001). Whether resistance to stress is enhanced or tumor incidence is reduced in calorie-restricted Ames dwarf mice is a question that is currently being studied.

Snell dwarf mice

Another dwarf mutant with extended longevity is the Snell dwarf mouse. Early reports regarding Snell dwarf longevity were conflicting. Confounding factors such as husbandry, environment, and genetic background were likely responsible for the discrepancies, as several laboratories did not observe the reduced lifespans reported by Fabris and co-workers (1972; Silberberg 1972; Bartke et al. 1977; Schneider 1976; Shire 1973). Longevity studies have reported that Snell dwarf mice live on average 42% longer than wild-type siblings (Flurkey et al. 2001, 2002). Snell dwarf mice, like their Ames dwarf counterparts, are GH-, prolactin- and TSH-deficient. However, their deficiencies result from a mutation in *Pit1* resulting in a non-functional protein (Li et al. 1990). *Pit1* is a transcription factor downstream of *Prop1* that directs fetal pituitary cell differentiation into somatotrophs, lactotrophs, and thyrotrophs. Similar phenotypically to the Ames dwarfs, these mice lack circulating levels of three anterior pituitary hormones resulting in significantly reduced body size, female sterility, and hypothyroidism (Bartke 1979). Some aspects of antioxidative defense have been addressed in Snell dwarf mice. Skin-derived fibroblasts from Snell mice are more resistant to multiple forms of cellular stress (Murakami et al. 2003). Percentage increases in LD₅₀ values were observed in dwarf mice over those of wild-type mice following exposure to UV light (45%), H₂O₂ (147%), paraquat (53%), cadmium (180%), and heat (102%). These studies included both oxidative and non-oxidative challenges indicating an overall increase in stress resistance. To further examine whether this resistance was limited to oxidative insults, these studies were repeated in the pres-

ence of ascorbic acid or N-acetyl cysteine (NAC; Salmon et al. 2005). Both antioxidants protected against H₂O₂, paraquat, and cadmium-mediated cell death. However, no protection from cell death caused by UV irradiation was observed. Snell dwarf mouse fibroblasts were also exposed to other forms of stress, namely actinomycin D and methyl methanesulfonate (MMS). Dwarf fibroblasts were significantly more resistant (60% increase in LD₅₀) to cell death induced by MMS than were fibroblasts from wild-type littermates (Salmon et al. 2005).

Madsen and co-workers (2004) took a different approach to studying oxidative stress resistance in Snell dwarf mice. These investigators challenged dwarf and wild-type mice with 3-nitropropionic acid, an inhibitor of succinate dehydrogenase (mitochondrial complex II), which causes free radical generation in tissues (Coles et al. 1979; Fu et al. 1995), and measured three kinase cascades in the MAP kinase pathway. Dwarf mice exhibited lower responses in the MEK-ERK kinase cascade and a lack of c-Jun Ser63 phosphorylation, suggesting altered management of oxidative stress in the long-lived dwarfs compared with the wild-type control mice. Although antioxidative defense enzymes have not been systematically examined in the Snell dwarf, an overall enhancement of this system is likely responsible for increased resistance to oxidative insult as found in phenotypically identical Ames dwarf mice.

At this time, gene expression studies in the Snell dwarf mice have contributed little information regarding stress resistance. In one study, it was shown that Snell dwarf liver tissue expressed less HSP70, HSP90, and HSP60 than was found in wild-type mice (Dozmorov et al. 2002). In addition, GSTp18 mRNA expression was lower in the dwarf than that in wild-type littermates.

Increased stress resistance may also contribute to differential neoplastic disease occurrence. GH/IGF-1-deficient Snell mice resist cancer development following administration of chemical carcinogens and exhibit reduced growth of transplanted tumors (Bielschowsky and Bielschowsky 1961; Chen et al. 1972; Rennels et al. 1965). Spontaneous tumors and severity are also reduced in hypopituitary dwarf mice (Flurkey et al. 2001).

Growth hormone receptor knockout mice

Growth hormone receptor knockout (GHR-KO) mice live 38–55% longer than their wild-type siblings (Coschigano et al. 2000). These mice lack functional GH receptors due to targeted gene disruption resulting in GH resistance and Laron dwarfism (Zhou et al. 1997). Characterization of stress resistance in these mice has revealed differences compared with Ames and Snell dwarf mice. Activity levels of GPX in kidney tissues were 25% higher in GHR-KO compared with wild-type mice (Hauck et al. 2002). Catalase and GPX activities, however, were decreased in the liver tissue of the GHR-KO mice. When challenged in vivo with paraquat, the wild-type males survived longer than the knockout males, while mean survival in female knockout and wild-type mice did not differ (Hauck et al. 2002). In agreement with data in other dwarf mutants, skin fibroblasts derived from GHR-KO mice were significantly more resistant to paraquat (47%), H₂O₂ (108%), and UV light (194%) compared with wild-type cells (Salmon et al. 2005). No differences in resistance to cadmium were observed. However, recent preliminary data show that GHR-KO mice have elevated kidney, liver, and heart metallothionein levels compared with wild-type mice (Swinscoe et al. 2006a). Differences in circulating hormone levels (KO: high GH vs. Ames and Snell dwarfs: non-detectable GH), genetic background, and tissue or cell type studies may partially explain the altered antioxidative defenses between these mutants.

Regarding gene expression, Al-Regaiey and colleagues (2005) reported an increase of nearly 50% in hepatic mRNA expression of SOD2 in GHR-KO mice presumably mediated via increased FOXO protein and decreased Akt expression. Increased activity of the FOXO transcription factor is suggestive of increased protection against cellular stress. Minimal differences were noted in liver gene expression patterns between GHR-KO and normal animals. In contrast, stress resistance-related gene expression differences were not observed between GHR-KO and normal mice (Miller et al. 2002). Similar to observations in Ames and Snell dwarf mice, the incidence of neoplastic disease is greatly

reduced in GHR-KO mice (Ikeno and Bartke, unpublished data).

Little mice

Mutation of the receptor for the GH-releasing hormone also causes dwarfism in mice and extends lifespan by 23–25% (Eicher and Beamer 1976; Flurkey et al. 2001). These mice, termed “little,” exhibit GH deficiency and live longer if placed on a low-fat diet (Flurkey et al. 2001). However, no information regarding stress resistance in these mice has been reported.

IGF-1 receptor heterozygous mice

Although IGF1-R^{-/-} knockout mice are not viable, the IGF1-R heterozygotes^{+/-} created by Holzenberger and co-workers (2003) have a 50% reduction in IGF-1 receptors in several tissues and hence reduced signaling through this pathway. Female heterozygotes displayed a 33% increase in lifespan, while males had only a 16% increase, which was not statistically significant. Resistance to oxidative stress was evaluated in murine embryonic fibroblast (MEF) cultures. Cells cultured from the IGF1-R heterozygous mice were more resistant to H₂O₂ than wild-type cells. Similarly, an increased resistance to paraquat toxicity in the heterozygous KOs was observed relative to wild-type mice; however, this difference in sensitivity was found only in females. A similar mutant, the IGF1-R *neo*^{-/-}, displays a more severe reduction in IGF1 receptor expression and exhibits a higher capacity to endure and recover from oxidant-induced lung injury than mice expressing normal levels of IGF1 receptors (Ahamed et al. 2005). No differences in tumor incidence between IGF1-R ^{+/-} and wild-type mice were observed (Holzenberger et al. 2003).

P66^{shc} mice

Growth hormone and IGF-1 downstream effector mutants have also been developed and express differences in longevity. An adaptor protein

called p66^{shc} lies downstream of the IGF-1 receptor and modulates a p53-dependent oxidative stress response pathway that regulates intracellular ROS levels and oxidative stress-induced apoptosis (Trinei et al. 2002). p66^{shc} couples receptor activation with downstream signaling molecules. Mice lacking expression of p66^{shc} live 30% longer than wild-type mice (Migliaccio et al. 1999). These mutant mice express high levels of catalase and MEFs exposed to H₂O₂ and UV radiation are more resistant to cell death than cells from wild-type mice (Trinei et al. 2002). A reduction in oxidative damage to nuclear and mitochondrial DNA in several tissues along with decreased plasma isoprostane levels in p66^{shc} mice is suggestive of increased protection against oxidative stress (Napoli et al. 2003). Resistance to oxidative stress is observed in different cell types (fibroblasts, endothelial cells, hematopoietic precursor cells) from p66^{shc} mice. Paraquat administration to p66^{shc-/-} mice results in longer survival times (40%) compared with wild-type control mice (Migliaccio et al. 1999; Napoli et al. 2003). Orsini and co-workers (2004) showed that p66^{shc} knockout mice have an increased resistance to in vivo ischemic lesions compared with wild-type mice via increased FOXO protein activity and decreased Akt levels (Zaccagnini et al. 2004).

FIRKO mice

Another line of mice was generated with a defect in the GH/IGF-1/insulin signaling pathway by Bluher and colleagues (2003). These fat insulin receptor knockout (FIRKO) mice express a mutated insulin receptor gene in adipose tissue only and exhibit 85–90% reduction in the insulin receptor protein in fat tissues. FIRKO mice live 18% longer than wild-type counterparts, have lower body weights (15–25%), and exhibit a 50% reduction in fat mass (Bluher et al. 2003). No alterations in stress resistance-related gene expression were detected following extensive microarray analysis of adipocytes from FIRKO and normal mice (Bluher et al. 2004). No other information has been reported concerning resistance to oxidative or other forms of stress in this line of mice.

α -MUPA transgenic mice

Transgenic mice created to overexpress α -MUPA (urokinase-type plasminogen activator) in the brain were shown to live 20% longer, consume 20% less food and weigh 20% less than normal control mice (Miskin and Masos 1997). These observations are similar to those found in calorie-restricted mice. In addition, serum IGF-1 levels were 70% of normal levels in the transgenic mice. uPA is an extracellular serine protease and is a central component of the fibrinolytic system implicated in tissue remodeling, brain development, and plasticity (Sumi et al. 1992; Masos and Miskin 1997).

With regard to stress resistance, GSH levels were 31% higher in α -MUPA mitochondria, but total GSH levels in liver homogenates did not differ from those in wild-type mice (Tirosh et al. 2003). These transgenics exhibit lower basal levels of liver mitochondrial SOD2 mRNA and protein, lower SOD2 activity levels, reduced lipid oxidation, and increased aconitase activity in mitochondria compared with wild-type mice. However, the mutant mice maintain the capacity to upregulate SOD2 upon challenge (Tirosh et al. 2005). The activity of liver GPX was not different between α -MUPA and wild-type mice. Oddly, when challenged with paraquat, these mice were more vulnerable compared with normal mice (90% versus 10% mortality; Tirosh et al. 2005).

Spontaneous and induced tumor development is altered in α -MUPA mice. The incidence of spontaneous lung adenomas was significantly lower in α -MUPA mice compared with wild-type animals. Similarly, young α -MUPA mice showed lower numbers of premalignant aberrant cryptic foci in the colon following dimethylhydrazine (DMH) injections and lower incidence of dermal and gastric tumors following dimethylbenzanthracene (DMBA) injections (Tirosh et al. 2003, 2004, 2005).

Klotho transgenic mice

The most recent addition to the growing list of mice with extended longevity is the Klotho transgenic mouse. Overexpression of the *KLOTHO*

gene results in a 20–30% increase in lifespan of males and 18–19% increases in females in two different transgenic lines (Kuroso et al. 2005). *KLOTHO* inhibits the hormonal signaling of insulin and IGF-1 receptors. Activity of *KLOTHO* apart from this signaling pathway remains to be determined. Convincing evidence is presented for the ability of Klotho transgenic mice to resist oxidative stress (Yamamoto et al. 2005). Administration of the Klotho protein to HeLa cells significantly suppressed paraquat-induced lipid oxidation and promoted survival of cells treated with paraquat. DNA damage in Klotho transgenic mice was half of that of wild-type mice. In addition, these transgenic mice survived longer than normal mice following in vivo paraquat challenge. Klotho is thought to bind to cell surface receptors inhibiting FOXO and promoting its translocation. The nuclear FOXO binds to the SOD2 promoter and upregulates expression of SOD2 protein. Mechanistic studies revealed that Klotho stimulation of several cell types downregulated Akt and FOXO1, FOXO3a, and FOXO4 phosphorylation. SOD2 mRNA levels were increased in muscle tissues of Klotho transgenic mice as were SOD2 protein levels (234%) relative to normal mice. These investigators concluded that Klotho suppresses aging by inhibiting IGF-1 signaling and increasing resistance to oxidative stress. Further studies utilizing the Klotho protein and signaling pathway will likely enhance the present understanding of the relationship between tolerance to stress and lifespan.

Insulin receptor mutant mice

Additional evidence that depressed insulin signaling leads to increased stress resistance is drawn from a recent report where leucine was substituted for Proline-1195 in the insulin receptor using knock-in techniques in mice (Baba et al. 2005). The heterozygous mice (IR mutants) showed suppressed kinase activity of the insulin receptor with normal growth. Under hyperoxic conditions (80% O₂) mutant female mice survived longer (33%) than wild-type females, while mutant males lived 18% longer than wild-type

males. Higher levels of MnSOD, along with increased survival under oxidative stress, supports the role of insulin/IGF-1 signaling in stress resistance. Application of calorie restriction to these mice further extended survival under oxidative conditions, suggesting potential independent mechanisms to enhance stress resistance. Unfortunately, no data regarding lifespan under normoxic conditions were reported as these studies are not yet completed.

Other mice with extended lifespans

Mice with extended lifespans that do not exhibit a direct link to GH/IGF-1/insulin signaling are far fewer in number. Many of these phenotypes target oxidative stress resistance specifically. Transgenic mice overexpressing mitochondrial catalase (MCAT) live 17–21% longer than wild-type mice (Schriner et al. 2005). Catalase activity was significantly higher in heart mitochondria, skeletal muscle, and heart tissues from MCAT transgenic mice compared with normal mice. In turn, cardiac pathology was significantly reduced in the MCAT mice. These MCAT mice generate 25% less heart mitochondrial H₂O₂ and H₂O₂-induced aconitase inactivation, suggesting increased protection from ROS. Oxidative DNA damage was also lower in skeletal muscle and heart tissues of MCAT mice as were the numbers of mitochondrial deletions in 21-month-old MCAT skeletal muscle. No information is available at this time regarding resistance to other forms of stress or tumor incidence in these mice.

Transgenic mice overexpressing thioredoxin (TRX-tg) have been used to evaluate the role of this protein in stress resistance and longevity. Thioredoxin is a redox-active protein with diverse biological activity including antioxidant, growth-promoting, anti-apoptotic, and inflammation-modulating properties. Thioredoxin transgenic mice exhibit lifespan extensions of 22–35% (Mitsui et al. 2002) and are relatively resistant to ischemia-mediated brain damage compared with control mice. Bone marrow cells from TRX-tg mice were more resistant to UV radiation than those from normal mice. A significant

body of literature has been recently reviewed regarding the role of TRX in human health and disease (Burke-Gaffney et al. 2005). Additional studies of TRX-tg mice and aging will be of great interest, as these animals are promising subjects for exploring the role of oxidative stress in aging and lifespan.

Mice heterozygous for $mClk1^{+/-}$, the murine homolog of *clk-1*, share similar characteristics with their nematode *clk-1* mutant counterparts (Liu et al. 2005). *Clk-1* is a factor involved in the biosynthetic pathway of ubiquinone (coenzyme Q). Although the complete knockout produces embryonic lethality, the heterozygous mice exhibit normal coenzyme Q levels and live 15–30% longer on average than wild-type mice (on at least two different backgrounds; Levavasseur et al. 2001; Liu et al. 2005). These investigators propose that a loss of heterozygosity occurs in old $mClk1^{+/-}$ mice. These old mice have reduced ubiquinone levels suggesting that the entire biosynthetic pathway of ubiquinone is turned off when *Clk1* is absent. Similar events have been reported in yeast (Marbois and Clarke 1996). They also reported that $mClk1$ knockout embryonic stem (ES) cells lack ubiquinone, produce low levels of basal and induced ROS, and resist ROS-induced apoptosis. Furthermore, these cells were resistant to other cellular stressors such as etoposide, anisomycin, all-trans retinoic acid, and serum withdrawal. Coupled with lower levels of ROS, $mClk1$ cells had significantly lower oxidative damage to DNA and lipids compared with normal cells. Similarly, liver tissue from heterozygote $mClk1$ mice also displayed less DNA damage relative to normal animals. Since ubiquinone can be considered both a pro-oxidant and an antioxidant, these data suggest that coenzyme Q may participate in creating stress, and thus reducing its levels may favor increased resistance to oxidative damage. The results of this study provide further evidence that resistance to oxidative stress accompanies longevity.

Calorie restriction and methionine restriction

For comparison, the effects of calorie restriction (CR) and methionine restriction (Meth-R) on

stress resistance will be addressed briefly. The life-extending effects of calorie restriction have been demonstrated in multiple animal species. Several studies have shown that CR lowers mitochondrial free radical generation in various tissues resulting in the reported decrease in oxidative damage to protein, DNA, and lipids. Examination of antioxidant defenses in CR animals yields conflicting data; some reports show increased antioxidant enzymes, while others have shown the opposite or that no discernible pattern has been observed (Luhtalta et al. 1994; Yu 1996; Lass et al. 1998; Judge et al. 2004; Sohal et al. 1994). However, CR increases non-enzymatic antioxidants; in particular, GSH (Armeni et al. 1998). Calorie restriction does increase resistance to other stressors such as heat, toxic drugs, and inflammation (Heydari et al. 1993; Klebanov et al. 1995; Berg et al. 1994; Duffy et al. 1995; Keenan et al. 1997). DNA and protein repair mechanisms have also been shown to be altered by CR, in directions beneficial to the animal (Guo et al. 1998; Lewis et al. 1985; Selsby et al. 2005; Stuart et al. 2004). All in all, the lifespan of calorie-restricted rodents is extended by mechanisms that likely involve resistance to stress, resulting in reduced cellular damage.

Limiting intake of a single amino acid, methionine, also extends the lifespan of mice (Miller et al. 2005). Longevity in rats on methionine-restricted diets was previously shown (Orentreich et al. 1993; Richie et al. 1994). In the most recent study, methionine restriction induced lower circulating IGF-1, thyroxine, insulin, and glucose levels compared with control-fed mice. Mice fed the methionine-restricted diet were significantly less sensitive to acetaminophen (an agent that causes oxidant-induced liver damage) compared with mice fed the control diet. An increase in stress resistance is thus observed following both CR and Meth-R, possibly involving similar mechanisms.

Discussion

Several questions arise regarding the relationship between stress resistance and longevity. What are the mechanisms responsible for the overall upre-

gulation in defense capacity in these mutant mice? Is the major player in longevity antioxidative defense, or do all the defense systems participate equally? Are these defense mechanisms turned on as a group (one major signal) to overcome adversity encountered in natural environments (e.g., nutrient availability, temperature, disease) or does each system have a different “switch”? Also, do the increases in protective measures at the cellular level influence lifespan significantly, or are there yet undiscovered pathways at play?

Disparity in growth and related metabolic consequences are observed between the mutant mouse strains described. This review does not consider whether or not metabolic rate is altered; rather, it weighs differences in the activation of signal transduction pathways and changes in metabolism of various substrates. Alterations in metabolism are oftentimes accompanied by changes in respiratory chain activity. A decrease in activity of the electron transport chain leads to lower ROS production and, when coupled with enhanced antioxidant defense capacity, to lower levels of oxidative damage. These changes are often concurrently reported in long-living mutant mice.

Evidence in yeast, worms, and flies suggests that growth factors and factors that affect nutrient storage activate kinases that downregulate stress resistance transcription factors and consequently catalase, SOD, and heat shock proteins (Longo and Fabrizio 2002). Based on current evidence, it appears that similar mechanisms may be operating in mammalian systems. With a disruption in growth factor signaling, the demand for protein synthesis is decreased throughout the postnatal period and reproductive maturity. This lower demand for protein synthesis may cause an overall shift in resources away from growth and proliferation and more toward defense or protective systems. Thus enters the FOXO family. FOXO transcription factors are perhaps the most direct molecular link between longevity, stress resistance, and nutrient signaling. Stimulation of IGF-1/insulin signaling occurs in times of nutrient abundance and during periods of growth, thus promoting cellular proliferation, growth, and energy storage (Datta et al. 1999; Kops et al.

2002; Tatar et al. 2003; Brunet et al. 2004). FOXO transcription is inhibited by IGF-1/insulin signaling. However, when nutrients are scarce, growth factors are deficient, oxidative stress is present or signaling is impaired via mutations in the IGF-1/insulin receptors or their downstream effectors, phosphorylation of FOXO is inhibited, and its nuclear translocation is promoted. Nuclear FOXO binds directly to the SOD2 promoter and upregulates its expression, thus conferring resistance to oxidative stress. Therefore, there is a metabolic shift away from growth toward cytoprotection.

Nuclear FOXO likely binds to other promoters and consequently alters gene expression. There are several reports indicating that factors other than those within the IGF-1/insulin/PI3K/Akt signaling pathway are involved in the regulation of FOXO activity, including but not limited to serum and glucocorticoid-inducible kinase (SGK), jun-N-terminal kinase (JNK), and β -catenin (Brunet et al. 2001; Essers et al. 2004, 2005; Wang et al. 2005). FOXO factors also appear to play a role in tumor suppression in a variety of cancers (Greer and Brunet 2005). Therefore, FOXO may be the factor that integrates nutritional and stress signals to regulate growth, cellular proliferation, and stress resistance (Accili and Arden 2004).

An upregulation of heat shock proteins prevents proteins from unfolding, thus maintaining conformation and activity. In addition, irreparably damaged proteins are hastened for destruction by these molecular chaperones. In the face of a decrease in protein synthesis due to a lack of stimulation (via impaired GH/IGF-1 signaling), an altered heat shock protein response would aid in the preservation of overall functional protein levels. Alterations in this system, among others in long-living mice, emphasize that the lower demand for growth (and in many cases reproduction) may direct valuable resources toward cytoprotection.

Resistance to oxidative stress is often coupled with increased tolerance to other types of stress, suggesting that there are multiple inputs that control regulation of protective mechanisms. Although only limited information regarding resistance to different types of stressors is avail-

able in the growing list of longevous mice, there are hints in the data that indicate that at least some of these mice are more resistant to several types of stressors than their wild-type counterparts. For example, the Ames dwarf mouse not only exhibits enhanced antioxidative defenses, but several of its tissues exhibit increases in heat shock proteins, metallothionein, methionine sulf-oxide reductase protein, and glutathione-S-transferase activities, which are known to maintain protein conformation and activity and to counter endogenous and exogenous toxins. Similarly, *in vitro* data in Ames, Snell, and Laron dwarf mice indicate resistance to multiple forms of stress (Murakami et al. 2003; Salmon et al. 2005). The degree of stimulation via the IGF-1/insulin pathway is reflected in differences in growth and lifespan among the different types of mutant mice (i.e., IGF-1R heterozygote versus dwarf strains) while the degree of stress resistance conferred via this pathway is more difficult to ascertain.

Antioxidants and phase II drug-metabolizing enzymes act together to detoxify and eliminate substances that induce chemical stress and result in cellular damage. Constitutive expression of these enzymes is low under physiological conditions. Studies have shown that expression of these protective proteins is increased upon exposure to stress. Several of these components comprise a defense system (that includes GSH biosynthetic proteins), the induction of which is controlled by the transcription factor NF-E2-related factor (Nrf2), which acts via the antioxidant response element (ARE) to activate transcription (reviewed in Nguyen et al. 2003; Chanas et al. 2002; McMahon et al. 2001). Nrf2-dependent ARE-driven genes include those involved in glutathione homeostasis (γ -glutamyl-cysteine synthetase [Gclc], GSTs, glutathione reductase, GPX), detoxification of H₂O₂ (thioredoxin, peroxiredoxin, catalase, SOD), iron homeostasis (heme-oxygenase-1, ferritin), growth factor signaling, and NADPH homeostasis (transketolase, malate, glucose-6-phosphate dehydrogenase; Lee et al. 2003), among others. The function of several Nrf2-dependent genes is reliant upon GSH, and the expression of these genes is important to maintain cellular redox homeostasis and prevent cellular damage. Protein phosphory-

lation pathways, including MAPK, PI3K, and PKC, have been implicated in the regulation of ARE response to chemical stress (Nguyen et al. 2003). Several studies have suggested that the contribution of the MAPK pathways in regulating the ARE is dependent upon the type of inducing agent (Yu et al. 1999, 2000a, 2000b; Alam et al. 2000; Masuya et al. 1998). Within the p38MAPK pathway specifically, PPAR-gamma coactivator-1 α (PGC-1 α) appears to be one of the major links between energy metabolism and cytoprotection, the activity of which is modified by extracellular signals (reviewed in Melloul and Stoffel 2004). The PI3K pathway has been shown to be important in transducing cellular responses to phenolic antioxidants and oxidative stress through the ARE (Kang et al. 2000, 2001; Lee et al. 2001, 2002). Experiments evaluating the role of the PKC pathway provided the first evidence of a direct effect of a kinase on the activation of the ARE in an Nrf2-dependent mechanism (Huang et al. 2000). The nature of the inducing agent, the cell type, and the context of the ARE in the gene are equally important factors that determine responses to cellular stress (Nguyen et al. 2003). Sulfhydryl chemistry may also be an important regulator of the ARE: the common factor linking compounds that induce ARE-driven transcription is that they can directly (or following metabolism) produce thiol-reactive compounds. Oxidative stress modifies cysteine residues on Nrf2 effector proteins and affects transactivation of the ARE (Itoh et al. 1999; Dhakshinamoorthy and Jaiswal 2001). Therefore, evidence exists of direct relationships and the potential for crosstalk between IGF-1/insulin signaling pathways and stress response pathways that affect the ability of organisms to react appropriately and counter stress effectively. The communication between these pathways is responsible for regulating cellular responses to growth-stimulating signals and stress responsiveness, and thus may serve as one of the major links between longevity and stress resistance.

Another potential candidate that integrates growth factor and nutrient signaling within the PI3K/Akt pathway is mammalian target of rapamycin (mTOR). Downregulation of the translation regulatory signaling pathways via PI3K/Akt/

mTOR in Ames mice provides evidence of the decreased protein synthesis, lack of growth, and, potentially, the decreased incidence of cancer in the face of increased food consumption (Sharp and Bartke 2005). In addition, the reduction in PI3K and mTOR signaling and subsequent attenuation of anabolism may simulate a stressful situation and upregulate general defense and repair systems, which, in the long term, extend lifespan.

A recent report showed that dwarf mice (including Ames, Laron, and Snell) exhibit constitutive expression of peroxisome proliferator-activated receptor α -regulated (PPAR- α) genes (Stauber et al. 2005). The PPARs are members of a nuclear receptor superfamily that are known to be involved in lipid metabolism, stress responses, and cardiovascular disease. Activation of these receptors by peroxisome proliferators (PP) increases the size and number of peroxisomes within the cell. Growth hormone has been shown to control the expression of many PP-regulated genes (Zhou and Waxman 1999a, 1999b; Zhou et al. 2002). The dwarf mutants display elevated levels of PPAR- α mRNA and protein findings that definitely assist in the overall understanding of tolerance to stress.

Finally, a small but growing body of literature exists regarding the role of “hormesis” in lifespan extension. Hormesis refers to the idea that low to moderate doses of typically detrimental agents (heat, radiation, chemicals) or stresses are beneficial (Furst 1987). Increased ability to cope with stressors, increased disease resistance, and increased longevity are considered beneficial responses or outcomes following exposure to such low-intensity insults. Agents with hormetic effects increase the activity of genes that increase defense mechanisms and protect cells from damage. There are multiple examples of this phenomenon (including calorie restriction) that may reflect an underlying physiological survival response that is conserved among species throughout evolution. Reduced signaling of the GH/IGF-1/insulin pathway in mice (and other species) leads to significant lifespan extension. Disturbing this hormonal system may be the hormetic mediator in these mutants that leads to the associated increase in stress resistance and resultant longevity.

Subtle differences between mutants, along with differences in the protocols used for evaluation of stress resistance, lead to some of the enormous differences reported in the literature. In addition, an interesting debate proposes that stress resistance is positively correlated with longevity only in stressful conditions, and that under non-stressful conditions, stimulating increased repair and defense capacity has yet to be strongly associated with extended longevity (Le Bourg 2002). A more systematic approach examining several mutants and utilizing similar endpoints will assist in the determination of degree of resistance in both stressful and non-stressful situations.

There appears to be an evolutionarily well-conserved mechanism that balances growth and metabolism with the cellular stress defenses that consequently regulate lifespan. FOXO may serve as the major translation program that coordinates physiological responses to environmental perturbation. The differences in longevity and stress resistance between long-living mutant mice result from complex interrelationships among pathways that coordinate these signals. Comparison of the mammalian system to other organisms utilized in aging research (fruit flies, nematodes, yeast) has and will continue to point toward conserved mechanisms common to many species. In addition, further investigation of stress resistance will suggest potential targets to create therapies to extend human health span, increase lifespan, and contribute to our collective understanding of the aging process.

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