The Regulation of Cellular Stress Resistance and DNA Repair in a Long-lived Mutant Mouse

by

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To my family and friends

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3-NPA	3-nitropropionic acid
4-HNE	4-hydroxyalkenals
6-4PP	pyrimidine (6-4) pyrimidone photoproducts
α-MUPA	murine urokinase-type plasminogen activator
AL	ad libitum
BSA	bovine serum albumin
BuA	sodium butyrate
CM	complete media
CPD	cyclobutane pyrimidine dimer
CR	caloric restriction
DD	dietary deprivation
DILP	*Drosophila* insulin-like peptides
DMBA	dimethylbenzanthracene
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
DR	dietary restriction
DSB	double strand break
ED50	effective dose 50%, median effective dose
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ER	endoplasmic reticulum
FCS	fetal calf serum
GFP	green fluorescent protein
GGR	global genome repair
GH	growth hormone
GHR	growth hormone receptor

GHR-KO	growth hormone receptor knockout
GpX	glutathione peroxidase
HSP	heat shock protein
IGF-I	insulin-like growth factor I
IFG-IR	insulin-like growth factor I receptor
JH	juvenile hormone
JNK	Jun N-terminal kinase
KO	knockout
LD50	lethal dose 50%, median lethal dose
MAPK	mitogen-activated protein kinase
MCAT	mitochondrial overexpression of human catalase
MDA	malonaldehyde
MEF	murine embryonic fibroblast
MEK	MAPK/ERK kinase
MMS	methyl methanesulfonate
MR	methionine restriction
NAC	N-acetylcysteine
NAD	nicotinamide adenine dinucleotide
nDNA	nuclear deoxyribonucleic acid
NER	nucleotide excision repair
NMR	naked mole-rat (*Heterocephalus glaber*)
mtDNA	mitochondrial deoxyribonucleic acid
oxo8dG	8-oxo-2-deoxyguanosine
PARP-1	poly(ADP-ribose) polymerase
PBA	4-phenylbtyrate
PBS	phosphate buffered saline
PCMT	protein carboxyl methyltransferase
PI3K	phosphatidyl inositol 3-kinase
PGL	propyl gallate
PTEN	phosphatase and tensin homolog
QPCR	quantitative polymerase chain reaction

RNAP	ribonucleic acid polymerase
ROS	reactive oxygen species
SD	serum-deprived
SOD	superoxide dismutase
Sod1	Copper-zinc superoxide dismutase
Sod2	Manganese superoxide dismutase
TCR	transcription coupled repair
TOR	target of rapamycin
TSA	tricostatin A
TSH	thyroid stimulating hormone
UDS	unscheduled DNA synthesis
UV	ultraviolet
WST-1	water-soluble tetrazolium

ABSTRACT

Mutations that extend lifespan in invertebrates often lead to resistance to multiple forms of stress, suggesting that stress resistance might also be important in lifespan determination in mammals. Work in this thesis supports this idea from several perspectives.

Snell dwarf mice have a mutation in the gene *Pit1* which extends their lifespan approximately 40% relative to controls and delays the progression of multiple age-related pathologies. We found that skin-derived fibroblast cultures grown from Snell dwarf mice are resistant to cell death induced by both oxidative stresses and stresses that are, in part, oxidation-independent, like heat, heavy metals, and DNA damaging agents. We also found that fibroblast resistance to the oxidative stressor peroxide is correlated with resistance to other stressors, both oxidative and non-oxidative, suggesting regulation of these properties by an overlapping set of mechanisms. We found similar patterns of resistance in fibroblasts grown from mice with other lifespan-extending mutations. An additional set of experiments showed that Snell dwarf fibroblasts have an enhanced ability to repair UV-induced DNA lesions, providing a possible explanation for their resistance to death cause by UV irradiation. Snell dwarf fibroblasts express higher levels of two nucleotide excision proteins than control, which may contribute to their enhanced DNA repair and to delayed aging and diminished neoplasia in these mice.

Extending these approaches to comparative biology, we found that resistance to some cellular stressors is correlated with maximum lifespan across mammalian species. Fibroblasts from long-lived species of rodents are significantly resistant to heavy metals and some oxidative stressors, and show similar trends for death caused by heat or DNA alkylation. For other agents however, such as UV light, there was no association between lifespan and cellular stress resistance. These results suggest that as rodent species evolved longer lifespan, there was also a coordinate increase in the cellular resistance to many, but not all, cellular stressors.

Overall, these results support the idea that mechanisms that regulate lifespan in mammals also tend to increase cellular stress resistance. The further study of the mechanisms of stress resistance in mammals may then help us better understand the molecular regulation of aging.

CHAPTER I

INTRODUCTION

Aging is a constant, progressive process wherein healthy, young adult organisms become frail, old organisms with a greater susceptibility to illness, injury and death (1). Under this definition, the aging process is thus presumed to begin following the attainment of maturity even as many of the phenotypes of aging are unable to be viewed until late in life. The aging process affects multiple organs, tissues and cell types and involves nearly every biochemical and physical function of the body that has been measured (2). In part because of the relative ease of observing the physical attributes of the aged, much is known about what defines an aged individual but little is known about the processes that regulate the aging process in early adult life. In addition, the extent of our knowledge of aged phenotypes has contributed to a problem in the study of biogerontology in that it is often quite difficult to distinguish the causes from the effects of aging. As such, it has been proposed that the goal of aging research should be the discrimination of cause and effect in an effort to narrow down the physiological regulatory mechanisms of the aging process (3).

Extended longevity and stress resistance in *C. elegans*

One of the most important and widely used models for studying the mechanisms of aging is the nematode *Caenorhabditis elegans*. Prior to the development of *C. elegans* as a biogerontological model, aging research was largely performed using comparative studies; that is, measuring how a particular phenotype differs between young and old organisms. The first report of a single gene mutation that could extend longevity in *C. elegans* represented a major paradigm shift. After this finding, it was possible to use genetic tools to dissect the potential mechanisms of the aging process.

Subsequent exploration into these mechanisms has hinted at the importance of the mechanisms of stress resistance in the regulation of longevity.

Stress resistance of long-lived mutant *C. elegans*

Klass was the first to report that mutations in *C. elegans* could extend lifespan (4). In this study, a screen for long-lived mutant worms generated by random mutagenesis produced 8 strains that lived greater than 50% longer than control worms. In further study of these worm lines, Friedman and Johnson found that the lifespan extension effect of three of these lines could be traced to mutations in a single gene they dubbed *age-1*, the first reported gerontogene (5). Later, another genetic mutant, a reduction of function mutant of the gene *daf-2*, was found to extend lifespan to a level more than two-fold that of control worms (6). Both *age-1* and *daf-2* mutations induce, at non-permissive temperatures, an alternate developmental pathway in which larvae enter a diapause state called the dauer (7). In wild-type worms, unfavorable or stressful conditions such as limited food supply and high population density favor dauer formation. During this time, dauer larvae are developmentally arrested and express sets of genes that protect them from harsh conditions such as elevated temperature, desiccation, oxidation and intense γ-radiation (7-9). Worms with mutations in *age-1* and *daf-2* undergo dauer formation at high temperatures (>35º C), but at permissive temperatures these mutations have little effect on fertility or the rate of development and reproduction (5;6;10;11). The lifespan extension of both *age-1* and *daf-2* mutants worms can be abolished by a mutation in either *daf-16* or *daf-18*, suggesting that the products of these two genes are required for the lifespan extension induced by the *age-1* and *daf-2* mutations (6;12). Further analysis of these genes has found that *age-1* encodes a homologue of the mammalian phosphatidyl inositol 3-kinase (PI3K), *daf-2* encodes an insulin receptor tyrosine kinase that is the worm homologue of the mammalian insulin receptor, *daf-18* encodes a homologue of mammalian phosphatase and tensin homolog (PTEN) which inhibits PI3K signaling, and *daf-16* encodes a protein that belongs to the forkhead family of transcription factors that respond to signals through the PI3K signaling pathway (13-16). The sequence similarities of these genes to those in the mammalian insulin/insulin-like growth factor I

(IGF-I) signaling pathway thus suggested that in *C. elegans,* diminishment of signals through the worm's insulin-like signaling pathway can extend longevity. Subsequent analysis of other members of this pathway have shown that they, too, can extend worm lifespan; loss of function mutations in *pdk-1* and *sgk-1* can extend lifespan almost two fold in *C. elegans* (17;18).

In addition to extending longevity, mutations in the *C. elegans* insulin-like signaling pathway often render worms relatively resistant to a variety of different lethal agents. When *age-1* mutants were cultured in the presence of the oxidizing agent hydrogen peroxide, they were found to live significantly longer than control worms (19). Subsequently, *age-1* mutant worms were found to be resistant to other oxidative stressors including paraquat, an herbicide that stimulates intrinsic reactive oxygen species production (20), and juglone, which causes intracellular redox cycling and superoxide production (21), and also resistant to culture at high oxygen concentrations (22). In addition, *age-1* mutants have been found to be resistant to agents that are thought to act, at least in part, independently of oxidative stress; *age-1* mutants are resistant to the lethal effects caused by UV light (23), to incubation at high temperature (24) and to incubation with the heavy metals cadmium or copper (25). Mutations in *daf-2* exhibit a similar pattern of stress resistance to all agents tested including an increased resistance to oxidative stress induced by paraquat or menadione (26), increased resistance to UV (23), increased resistance to heat (27), and increased resistance to cadmium and copper (25). Similar to their effects on lifespan extension, mutants of *daf-16* can suppress the stress resistance effects of these mutations. Double mutants for *age-1:daf-16* or *daf-2:daf-16* are no more resistant to UV than control worms (23). Similarly, *age-1:daf-16* worms are not resistant to either cadmium or copper ions (*daf-2:daf-16* were not tested) (25). Furthermore, neither *daf-2:daf-16* nor *age-1:daf-16* worms exhibit resistance to paraquat (26;28). The loss of stress resistance with mutation in *daf-16* probably results from the loss of *daf-16* mediated transcription of several stress defense proteins (29). Other genes in the insulin-like signaling pathway can also alter stress resistance in a *daf-16* dependent manner. For example, *sgk-1* mutant worms are resistant to paraquat, peroxide, and heat (18), and *pdk-1* mutant worms are resistant to paraquat and heat (28). At least two downstream targets of *daf-16* have been implicated in the regulation of longevity and

stress resistance. Overexpression of *old-1*, a stress-inducible tyrosine kinase, extends worm longevity and renders worms resistant to heat and UV light but requires the activity of *daf-16* to cause both phenotypes (30). Similarly, *scl-1* is a putative secretory protein under the regulation of *daf-16,* and its activity is required for *age-1* and *daf-2* extension of longevity. Knockdown of *scl-1* function by RNA interference extends longevity and confers resistance to heat and UV, phenotypes that are lost with the loss of *daf-16* (31). These data thus suggest clearly that diminished signals through the worm insulin-like signaling pathway can both extend longevity and induce resistance to multiple forms of stress.

The gene product of *daf-16* also plays a role in regulation of stress resistance and longevity associated with mutations of genes that are not directly involved in insulin-like signaling, thus suggesting a convergence of pathways that depend on this forkhead family transcription factor. For instance, the *C. elegans* homologue of mammalian Jun N-terminal kinase (JNK) can extend the longevity of worms when its activity is increased by overexpression of *jnk-1* itself or by positive regulators of JNK signaling (32). This pathway seems to involve regulation of *daf-16*, because in response to heat stress, JNK directly promotes the translocation of *daf-16* from the cytoplasm to the nucleus (32). A mutation in *spe-26* results in decreased sperm production in *C. elegans*, extends worm lifespan 65% (33) and generates worms that are resistant to the effects of heat and UV light (23;27). The longevity extension and stress resistance of *spe-26* mutant worms can be suppressed by mutations in *daf-16* (23). Mutations in the *clk-1* gene, which is important for ubiquinone biosynthesis and cellular respiration, have also been shown to extend lifespan and render worms relatively resistant to UV irradiation (23;34). Some have suggested that the lifespan extension of *clk-1* is independent of *daf-16* activity (35) while in contrast others have suggested that lifespan and UV resistance may be dependent on *daf-16* function (23;36). The gene *sir-2* encodes a histone deacetylase that modulates chromatin silencing and requires nicotinamide adenine dinucleotide (NAD) as a cofactor. Overexpression of *sir-2* extends *C. elegans* lifespan in a *daf-16* dependent manner (37). Overexpression of *sir-2* also renders worms relatively resistant to paraquat and heat (38). Recent evidence suggests that the extended longevity of *sir-2* mutants is dependent on *sir-2* binding to *daf-16* through an interaction with 14-3-3 proteins, highly conserved

small acidic proteins that bind phosphoserine and phosphothreonine residues in a context-specific manner (38;39).

In *C. elegans,* extension of longevity and increased stress resistance might be, in part, due to expression of genes required for dauer formation. As mentioned above, dauer larvae represent an alternate worm phenotype designed to protect the worms from unfavorable conditions. Dauer animals are resistant to death induced by a host of agents including high heat, oxidation and radiation (7-9). Metamorphosis into the dauer form involves upregulation of a number of cellular defenses associated with stress resistance including the antioxidants superoxide dismutase and catalase and the heat shock proteins HSP70 and HSP90 (8;19;20;40;41). Many of these same proteins are upregulated in long-lived worms with mutations in the insulin-like signaling pathway. Mutant *age-1* and *daf-2* worms exhibit higher levels of superoxide dismutase and catalase activity than control worms (19;20) and also express higher levels of the heat shock protein HSP16 (42). However, HSP90 expression is low in adult mutant worms, in contrast to its high expression in dauer (43). Thus, it may be that longevity and stress resistance through insulin-like signaling are regulated in part by the same pathways that control dauer formation, but there must be some independence (44;45).

Genetic alterations in other pathways can also regulate *C. elegans* longevity and resistance to stress independently of *daf-16*. The group of enzymes known as TOR (Target of rapamycin) kinases regulates cell growth and proliferation in response to nutrients and mitogenic signals. The deletion of TOR kinase activity can more than double the lifespan of *C. elegans* even in the absence of *daf-16* activity (46). Worms without TOR kinase activity are also heat resistant (47). TOR kinases act, in part, regulating the synthesis of a subset of proteins. A similar effect is caused by loss of a specific eIF4E isoform (*ife-2*), a eukaryotic translation initiation factor that affects the rate of cap-dependent protein synthesis. Knockdown of *ife-2* in worms by RNA interference reduces global protein synthesis, protects from oxidative stress induced by paraquat or sodium azide and extends lifespan (48). These effects are independent of *daf-16* and can further extend the lifespan of *age-1* and *daf-2* mutants (48). Inactivation of the genes encoding CID-1, CHK-1 or CDC-25 by RNA interference can extend worm lifespan from 25-40% depending on the inactive gene (49). These proteins are regulators

of cell-cycle progression during *C. elegans* embryonic development. Knockdown of any of these genes also renders worms relatively thermotolerant and neither this effect, or the effect on longevity, require the activity of *daf-16* (49). Of these pathways presented in this section, all may play some role in detection of suboptimal conditions for growth and reproduction; TOR kinase activity is diminished in the presence of low nutrient levels, and in turn low nutrient levels diminish protein synthesis and cell division. It may then be that evolution has led to the development of pathways that induce stress resistance and lower protein production in response to low nutrient levels.

Longevity of stress-resistant *C. elegans* mutants

Since long-lived worm mutants tend to be resistant to stress, the next question to be addressed is whether increased stress resistance is by itself sufficient to extend longevity in *C. elegans*. One way the relationship between stress resistance and longevity has been examined is by testing whether selection for resistance to stress can result in an organism that is longer-lived than those that had not undergone selection. For example, juglone is a compound that affects *C. elegans* intracellular redox cycling such that superoxide radicals are generated; most worms that are cultured in media containing juglone will not survive (50). In work by de Castro et al. (51), several lines of worms were generated from founders that survived juglone selection. These researchers found that this selection process produces lines of worms that were resistant not only to juglone, but also to heat, UV light, and hypoxia and to the toxicity of *Pseudomonas aeruginosa.* These data suggest that the stress resistance pathways that protect from juglone may be shared with those for other types of stressors, such as UV and heat, and that selection for resistance to one stress may then positively affect resistance to others. Further, these researchers found that this selection regime extended lifespan up to about 30% in most lines tested; some selected lines were stress resistant but not long-lived. In a similar line of investigation, selection for resistance to paraquat generated three worm strains that also tended to be resistant to heat and UV (52). One of the three resistant worm lines was found to be long-lived relative to control. Several groups have shown that selection for resistance to incubation at high temperature can also generate long-lived lines of worms

(53-55). Unfortunately these reports made no mention of whether these selected lines were also resistant to stresses other than heat, except for one report that long-lived, thermotolerant larvae were resistant to starvation-induced death (55). Similar to the results found for juglone and paraquat selection, not all lines generated by selection for heat resistance were long-lived. From these data, it seems clear that selection for stress-resistant *C. elegans* also tends to select for longevity in worms, though not all selected strains are long-lived. This was also seen in findings from Lithgow et al. (27) in which several heat resistant mutant worm strains, but not all, were long-lived. Thus, it seems that these experiments provide support for the idea that there is at least a partial overlap in the mechanisms that regulate aging and those that regulate resistance to stress.

There is also evidence that *C. elegans* can be rendered stress resistant and long-lived through a non-genetic process known as hormesis. Under hormesis, longevity is extended by exposing worms to a mild stress early in life. Several groups have shown that worms exposed to high temperature as young adults have lifespans greater than control worms and are also more resistant to heat (27;56-59). It is thought that hormesis activates genes that function to repair molecular damage, such as heat-shock proteins (60;61). The expression of these proteins may then protect cells from the damage produced by subsequent stress, thereby increasing thermotolerance, and perhaps prepare them to resist multiple cellular insults throughout life and repair age-related accumulative damage, thereby increasing longevity. Extended lifespan due to hormesis induced by other agents has also been shown. Pretreatment of worms with hyperoxia induces resistance to hyperoxia as well as juglone resistance, and also extends lifespan about 20% (59). In the same study pretreatment with juglone conferred juglone resistance, but unexpectedly did not extend lifespan. It was also reported that low doses of γ-irradiation from a ^{137}Cs source could extend lifespan to a small degree (62). However, these researchers were unable to replicate this effect (59). Pretreatment with UV light did not change UV resistance or lifespan (59). While exposure to high temperature early in life has consistently been shown to increase lifespan, results using other agents have been far more inconsistent. Thus, these findings suggest that heat-induced augmentation of protective pathways can extend life span, but may be unlikely to provide clues as to how lifespan and stress resistance are regulated in genetic mutations.

Calorie restriction, longevity and stress resistance in *C. elegans*

For nearly 60 years, it has been known that the lifespan of rodents can be extended by limiting caloric intake by approximately 1/3 that consumed by control animals (calorie restriction, CR) (63). This extension is accompanied by CR-mediated delay of age-related changes in numerous physiological, biochemical and pathological processes (reviewed in (64)). Studies of CR in other laboratory models have shown that many species respond to low calorie diets by increased lifespan, although there are exceptions (reviewed in (64)). Studies of CR using *C. elegans* have been encumbered by several technical issues. One problem is that *E. coli*, the usual laboratory food source for *C. elegans*, is toxic to some degree, and worm lifespan can be extended by providing *E. coli* killed by UV light or antibiotics (64, 65). Therefore, CR-mediated lifespan extension may in part represent reduced intake of *E. coli* associated toxins. A second issue is the difficulty in quantifying nematode food intake; it is quite easy to see if mice or rats have eaten all of their allotted diet, but less simple to evaluate consumption of *E. coli* on an agar dish or in a liquid culture. Because it is unclear whether the methods used to study CR in *C. elegans* are actually restricting caloric intake, they are often termed dietary restriction (DR).

A number of different methods have been utilized to explore the effects of DR on *C. elegans*. All present complications for interpretation. The first method tested involved reduction in the number of bacteria available to the nematodes. In a report by Klass (65), newly hatched *C. elegans* were placed in liquid cultures containing concentrations of bacteria from 0 to the conventional culture level of 1×10^{10} bacterium/ml and assayed for lifespan. This report showed that worms grown in the presence of bacteria at a concentration of 10^8 had a lifespan nearly double that of controls grown at a conventional bacterial concentration (10^{10}). Another report reduced the number of bacteria available on agar plates and found a similar DR-mediated lifespan extension (66). The problem with these reports is the issue of food source toxicity, so it is unclear if extension of lifespan is due to DR directly. Stress resistance was not evaluated in either study.

A second method for DR in *C. elegans* exploits mutations that diminish food intake. Feeding in *C. elegans* requires peristaltic ("pumping") contractions of the pharynx. Mutations in the gene *eat-1* cause a defect in the ability of *C. elegans* to perform these muscle contractions, thereby reducing intake of food (67). There are several different mutations of this gene that have been characterized, and many of them are long-lived; in fact, mutations of *eat-1* tend to extend lifespan of *C. elegans* to a greater degree than any other lifespan extending single gene mutant (68). However, the diminished food uptake in this model may trigger expression of the dauer phenotype, complicating interpretation of lifespan data. Unfortunately, stress resistance of *eat-1* mutants has not been characterized, but a similar mutation in *eat-2* seems to render worms resistant to heat (69).

A third *C. elegans* DR method has been derived which utilizes a semi-defined, sterile liquid media (axenic media). *C. elegans* cultured in axenic media were shown to live nearly two-fold longer than worms grown in conventional, bacteria-containing media (69;70). These studies also found that worms cultured in axenic media were thermotolerant relative to control worms. Additionally, DR increased the activities of the antioxidants superoxide dismutase and catalase, but strangely, DR worms were not resistant to paraquat or hydrogen peroxide relative to worms grown in culture media containing *E. coli* (69;70). Again, a primary issue with these studies is that control "ad-libitum"-fed worms were grown in media with bacteria making it difficult to interpret how much of the lifespan extension is due simply to diminishing the toxicity of the media. In addition, the growth, development, and metabolic rate of *C. elegans* are severely reduced when cultured in axenic media, further complicating the interpretation of the lifespan results (71).

Lastly, two groups have recently shown that removing adult *C. elegans* from their bacterial food source altogether can extend lifespan. This DR regime, more appropriately termed dietary deprivation (DD), might reduce effects of developmental delay on lifespan because food is only removed from the worms after they have reached adulthood. By simply transferring newly-adult worms to agar plates without a bacterial food source, both independent research groups have shown that *C. elegans* lifespan can be extended by about 30-50% (72;73). Further, these groups have found that DD increases

thermotolerance and resistance to oxidative stress of worms relative to those grown using standard conditions, i.e. with bacteria present. Although both groups used dead *E. coli* for the standard diet, the effects of residual toxicity cannot be completely eliminated. These researchers suggest that DD acts independently of dauer formation.

In general, DR of *C. elegans* represents a complex methodology in which it is oftentimes difficult to determine the effects of DR directly. There is some evidence that DR, however induced, may increase stress resistance in worms, but the evidence is still fragmentary.

Pharmacological extension of lifespan in *C. elegans*

There is growing interest in pharmacological agents that may confer many of the health benefits of genetic mutations that extend longevity or of calorie restriction. Some of the pharmacological agents tested for lifespan extending properties are thought to enhance stress resistance. For instance, EUK-134 and EUK-8 are antioxidant mimetics and have catalytic activities similar to those of the antioxidants superoxide dismutase (which converts oxygen free radicals to peroxide) and catalase (which converts peroxide to water and free oxygen). The dietary treatment of adult *C. elegans* with either EUK-134 or EUK-8 has been shown to increase both the mean and maximum lifespan without affecting growth rate of reducing fertility (74). In this same study, these researchers suggested that EUK-134 or EUK-8 diminished the effects of oxidative stress, because these agents increased lifespan of a *C. elegans* mutant with a dysfunctional electron transport chain. Further, treatment of nematodes with these agents renders animals resistant to death caused by paraquat, which causes the intrinsic formation of superoxide radicals, and also resistant to heat (75). However, a second group of researchers found no evidence that EUK-134 or EUK-8 could extend lifespan of adult *C. elegans*, and in fact suggested they are toxic at higher doses (76;77), despite the ability of these compounds to protect from paraquat-induced death. It is unclear why these groups have produced contrasting results, but since both found that EUK-134 and EUK-8 treatment conferred paraquat resistance, differences in longevity might be explained by variations in long-term culture conditions. Treatment with another antioxidant, Vitamin E, has also been

shown to extend lifespan in *C. elegans,* but the lifespan data were fragmentary and inconsistent (78). In this study, only one of the tested concentrations of Vitamin E positively affected lifespan; lower doses had no effect, higher doses were toxic and treatment in conjunction with other antioxidants had no effect. In a separate report, application of an isoform of Vitamin E was shown to extend nematode lifespan, reduce oxidative damage with aging and protect from death caused by UVB light (79).

There have also been several reports of longevity extension and increased stress resistance with compounds that have not been classified as antioxidants. Treatment with a complex mixture of polyphenol compounds isolated from blueberries extends *C. elegans* lifespan and also makes these worms resistant to high temperature (80). However, this treatment did not increase resistance to oxidative stress caused by either hydrogen peroxide or paraquat. Interestingly, treatment did reduce age-related accumulation of lipofuscin, a measure of intrinsic oxidative damage, perhaps suggesting a reduction of chronic oxidative stress. Extracts from *Ginko biloba* have a range of activities, including free radical scavenging (81). Treatment with one such extract, EGb 761, can extend *C. elegans* lifespan as well as confer thermotolerance and resistance to oxidative stress (82). Within the same report it was shown that a single component of the extract, the flavonoid tamarixetin, was sufficient to cause these effects.

A compound that has recently generated much excitement in gerontology is resveratrol, a phytoalexin produced by several plants and found in high levels in the skin of red grapes. Resveratrol has been shown to extend lifespan across many animal species from yeast to fish (83-85). The mechanism of resveratrol activity is still in contention, but may involve activation of sirtuin proteins, a class of NAD-dependent protein deacetylases thought to regulate many cellular processes including stress resistance (86). Recently, it was shown that resveratrol treatment can render *C. elegans* resistant to paraquat, but did not protect worms from heat (87).

Overall, the lifespan extending properties of most of these compounds is still under debate. While most of these compounds have also been shown to enhance resistance to some stressors, there is a lack of a consistent response. Thus, it is unclear if compounds that extend worm lifespan do so solely by increasing resistance to stress.

Biochemistry of long-lived *C. elegans*

It might be speculated that long-lived *C. elegans* mutants tend to be resistant to stress because these mutations also affect the activity of biochemical pathways that protect from cellular stress. For instance, the antioxidant activity of catalase and superoxide dismutase (all isoforms assessed together) is greater in *age-1* mutant worms (19;20). Also, the expression of the antioxidant and chelator metallothionein is higher in both *daf-2* and *age-1* mutant worms in comparison to their relative controls (25). However, the activity of these antioxidants does not address whether there are plausible hypotheses that could explain the increased resistance of long-lived mutant worms, not just to a single form of lethal stress, but to multiple forms of stress at the same time.

Heat shock proteins (HSPs) play a well-established role in protecting cells from the many misfolded proteins that commonly arise following heat shock and other types of stressors; these misfolded proteins, if not removed, can lead to cell death (88;89). HSP-16 is not expressed at appreciable levels in young adults of *C. elegans* (90), but can be induced by several stresses, especially by exposure to high temperature (91;92). Therefore, the level of HSP-16 after exposure to heat provides an indirect measure of a worm's ability to respond to stress. HSP-16 is expressed at higher levels in thermotolerant, long-lived *daf-2* and *age-1* mutant worms following thermal stress (42) and expression of extra copies of HSP-16 can confer resistance to heat and extend lifespan of worms by about 10% (93). In an interesting set of experiments, a reporter construct for HSP-16 linked to green fluorescent protein (GFP) was introduced into *C. elegans* to explore the effect non-genetic and stochastic (chance) variation has on stress resistance and thermotolerance. When worms expressing this construct are exposed to heat stress, or oxidative stress caused by juglone or paraquat, expression of HSP-16-GFP is induced (91). To measure the effects of chance on lifespan, individual isogenic worms carrying this construct were tested for HSP-16 expression following heat stress by assessment of GFP fluorescence. Fluorescence based sorting methods were used to divide the original worm population into subsets that differed in the level of reporter gene expression after short heat exposure, and worms in these classes then evaluated for lifespan and in some cases thermotolerance. Individual worms with high HSP response

lived longer than worms with lower response to stress (94;95). Worms with a high response also were more resistant to a subsequent lethal heat stress than those with a low response (94). These researchers suggest that HSP-16 itself is probably not responsible for the observed differences in survival but instead may reflect a physiological state that dictates the ability of an organism to deal with the rigors of living, perhaps by transcription of genes that have effects on aging (96).

A similar line of experiments has focused on the role of HSF-1, a transcription factor that regulates the transcription of stress responsive genes like *Hsp-16* following stress. Hsu et al. found that HSP-16 induction was dependent on HSF-1 and that overexpression of HSF-1 could extend worm lifespan about 40% (97). They also found that extra copies of HSF-1 rendered these worms resistant to heat and to paraquat-induced oxidative stress. Morley and Morimoto confirmed the life-extension by overexpression of HSF-1 and also showed that loss of HSF-1 in *age-1* and *daf-2* mutants suppresses their longevity to that of control (98;99). However, this result is complicated to interpret, because diminished survival in these forms may in part be due to the toxicity of accumulating aggregates of misfolded proteins (98;100). Overexpression of the gene that encodes *C. elegans* HSP-70 protein has also been shown to extend worm lifespan by about 40%, but the stress resistance of these worms was not reported (101).

These results show that lifespan can be extended by the increased expression of at least two HSPs and of a pathway that regulates HSP expression. The increased expression of the HSPs also has been shown to increase resistance to multiple forms of stress. Further, regulation of lifespan in other long-lived *C. elegans* mutants, such as *age-1* and *daf-2* mutants, seems to be dependent on the expression of HSPs. These results then suggest one biochemical mechanism that explains why mutations that extend lifespan in *C. elegans* also tend to render worms resistant to many types of stress.

Extended longevity and stress resistance in *Drosophila*

Drosophila melanogaster has had a long history as a laboratory model for the study of genetics and development. Researchers have been slow to use *Drosophila* as a model organism to study the mechanisms of aging, but there are many benefits of using

the fruit fly for studying aging. Like *C. elegans*, *Drosophila* have a relatively short lifespan and are easy to maintain in the laboratory. In addition, *Drosophila* researchers have discovered many interventions that can extend longevity, can call upon information from the completely sequenced genome, and have taken advantage of powerful molecular genetic techniques that have had decades of refinement. Experiments using *Drosophila* to explore the relationship between longevity and stress resistance provide a foundation for some of the experiments presented in this thesis.

Stress resistance of long-lived mutant *Drosophila*

A number of experimental interventions affect lifespan in Drosophila, including changes in temperature or physical activity, selection for longevity, genetic manipulation, or induction of hormetic effects by brief stress exposure. However, many of these interventions might exert much of their effects by modulating reproductive status, particularly in female flies. Reproduction itself often reduces lifespan in *Drosophila* (102;103), the so-called "cost of reproduction". Virgin females can live up to twice as long as females that have mated (104). The effects of reproduction on lifespan may reflect differential allocation of energetic resources away from somatic maintenance (105;106), or, more directly, by induction of somatic mutations (107). Exposure of female flies to seminal fluid products produced by the male accessory gland can increase female death rate (108;109). Stimulation of reproduction can also diminish resistance to oxidative stress. Female flies treated with methoprene, a juvenile hormone (JH) analogue that increases reproductive output by stimulating oocyte maturation, are significantly more sensitive to the toxic effects of paraquat; sterile females given the same treatment show no diminution of paraquat resistance (110;111). In the wild, flies undergo a reproductive diapause under poor environmental conditions during which vitellogenesis, or egg formation, is halted at a precursor stage due to decreased production of JH. In many ways, inhibition of fly reproductive status is akin to the dauer phenotype in *C. elegans*. For these reasons, studies of interventions that affect longevity and stress resistance in *Drosophila*, particularly in reproductive females, require careful interpretation. Thus, stronger support for lifespan-extending interventions may come

from performing such experiments in both reproductive and non-reproductive individuals, either by maintaining virginity or using female-sterile fly strains, and in males.

Some of the earliest experiments to investigate the genetics of aging and longevity involved laboratory selection in *Drosophila*. Three independent studies have shown that long-lived fly strains can be generated by selecting for late-life reproduction (112-114). The basic selection regime of these studies generated two classes of fly lines; "Young" lines were generated by using eggs laid by young adults while "Old" lines were generated from old adults which have had some time to reproduce prior to egg collection for the next generation. Since many flies die before the age at which eggs were collected in the "Old" lines, these lines show the combined effects of selection for late fecundity and adult survival. The lines generated by Rose (112) showed that selection for late-life fecundity over 15 generations could extend both the mean and maximum longevity of male and female flies of five independent "Old" lines relative to flies from five "Young" lines that had not undergone the same selection protocol. Similar results were shown for similar lines of flies generated by other research groups (113;114). Some have suggested that this protocol has inadvertently selected for diminished fecundity because "Old" lines are less fecund in early life, and suggested that this may explain, in part, the extended longevity of female flies in the "Old" lines. However, reproductive output, averaged over the entire lifespan, tends to be at least as high in lines selected for late fecundity as in unselected control lines (112) suggesting that the selective process did not lead to an overall difference in reproductive output. Further analysis of long-lived "Old" lines has also shown that these flies are resistant to the lethal effects of oxidative stress and heat. The mean survival in response to paraquat of the five "Old" lines generated by Rose is significantly greater than the survival of the corresponding "Young" lines (115). The lines generated by Luckinbill et al. displayed a similar pattern of paraquat resistance and were also found to be resistant to heat (113;116). Thus, these results suggest that genetic selection for extended longevity leads to a correlated selection response for genes that confer stress resistance.

The finding in *C. elegans* that a single gene mutation could extend lifespan (5) has stimulated many new studies using genetic approaches to explore ideas about aging

in flies as well. Genetic approaches can be used to confirm pre-existing hypothesis using candidate gene approaches, or be used to generate new hypotheses using random single-gene mutation screens. While *Drosophila* are particularly apt for random approaches due to the well-developed molecular genetic tools in the system and the relatively short lifespan of flies, only a few long-lived mutant fly strains have been developed in this manner. Flies with a mutation of the gene *methuselah (mth)*, which encodes a G protein-coupled transmembrane, receptor-like molecule, emerged from a screen for long-lived mutants among randomly mutated flies. *Mth* mutations can extend lifespan of both male and female flies by about 35% without reduction of fecundity (117). Mutation of *mth* also renders flies resistant to lethality induced by heat and by paraquat (117). The function of *mth* is still not clear, but mutations of the endogenous ligands of the gene product of *mth* also extend lifespan about 50% compared to control, and render flies relatively resistant to paraquat (118). Flies expressing a peptide ligand inhibitor of *mth* have recently been shown to live 30% longer than controls, suggesting that search for pharmacological agents that affect *mth* could be informative (119).

A second long-lived fly strain generated by random mutagenesis has a mutation in the gene *I'm not dead yet (Indy); Indy* flies have an average lifespan nearly two-fold that of control flies (120). Similar to *met* mutant flies, *Indy* flies are resistant to paraquat toxicity (121). The *Indy* gene encodes a dicarboxylate transporter which is thought to be involved in the transport of metabolites from the gut into nutrient storage sites and metabolic tissues such as the fat body, but mutations of *Indy* do not decrease basal metabolic rate, do not affect physiological measures such as flight or activity and do not reduce fecundity under normal laboratory rearing conditions (120;122;123). Thus, even though the genes products of *mth* and *Indy* have not been reported to modulate stress resistance directly, mutation of these genes can render flies resistant to stress and extend longevity.

Analysis of *C. elegans* mutants has suggested that pathways homologous to mammalian insulin and IGF-I signals have an important role in regulating longevity and stress resistance. Work in *Drosophila* has led to similar conclusions. Mutation in the insulin receptor gene (*InR*) yields dwarf flies that live about 85% longer than control for females and 50% longer for males (124). At least part of the sex difference in longevity

might be regulated by low JH and resultant diminished fecundity; treatment of females with a JH analogue restores fecundity and diminishes lifespan extension (124). Some mutations of an insulin receptor substrate *(chico)* also yield long-lived dwarf flies; homozygous *chico* mutants live up to 48% longer than control, and *chico* heterozygous flies live up to 36% longer than control (125). While female *chico* homozygotes are sterile, these flies live even longer than other sterile mutants on similar genetic backgrounds, suggesting that longevity extension of *chico* mutants may be at least partly independent of alterations in fecundity (125). For *chico* and *InR* mutants, age-related changes to the heart, including stress-induced failure, are reduced suggesting that these mutations can delay the progression of age-related pathologies (126). But the *chico* mutation does not seem to render flies stress resistant; female *chico* heterozygotes and homozygotes are not resistant to heat or paraquat relative to control flies, and male *chico* homozygotes are more sensitive than controls to these stresses (125). Further, larvae with loss of function *chico* mutations are sensitive to γ-irradiation as measured by eclosion of larvae to adult, though this may represent the adverse effects of reduced insulin signaling on eclosion more than stress resistance (127). Diminishment of insulin-like signaling can also extend lifespan through ablation of the neuroendocrine cells that produce *Drosophila* insulin-like peptides (DILPs) (128). In this study, cells were ablated in adult flies by tissue-specific expression of a pro-apoptotic gene. Loss of these cells reduced expression of neuroendocrine-specific DILPs and extended lifespan 10-35% depending on sex and reproductive status. Unlike *chico* mutants, these flies are resistant to lethality induced by heat or paraquat (128). In *C. elegans*, the longevity extending effects of diminished insulin-like signaling require the activity of the forkhead transcription factor *daf-16*. The *Drosophila* homologue of *daf-16* is *dFOXO* which has recently been shown to extend lifespan 20-50% and confer resistance to paraquat when overexpressed in the fat body of adult flies (129;130). In addition, flies overexpressing *d4E-BP*, a regulatory element of the *dFOXO* downstream effector eIF4E, are long-lived and peroxide resistant (131). In a related line of investigation, enhancement of JNK signaling, and subsequent antagonism of insulin-like signaling, by overexpression of a JNK-specific phosphatase, was shown to extend fly longevity up to about 30% and to increase resistance to paraquat (132). This activity of JNK was subsequently found to be

dependent on the activity of *dFOXO* which localizes to the cell nucleus in cells where insulin signals are low (133). Thus, these mutants show that lifespan extension when insulin/IGF-I-like signaling is diminished is conserved across species. It is unclear however why inhibition of the same signaling pathway has contrasting effects on stress resistance in some mutants, like *chico*. It may be that diminution of signals at any point in the pathway may have a positive effect on lifespan, but that stress resistance may be dependent on the actions of upstream genes such as *chico* in an insulin signaling-independent manner.

Longevity of stress resistant *Drosophila* mutants

In *C. elegans*, the argument for stress resistance as a determinant of lifespan is strengthened by numerous reports that worms selected or genetically altered to be stress resistant also tend to be long-lived. The results from similar experiments in *Drosophila* are less consistent, in some cases depending on the methodology for generating flies that are stress resistant. For instance, fly lines that had undergone selection for resistance to heat are thermotolerant, but not long-lived (134). A similar result was found for flies selected to be cold-resistant (134). However, flies lines generated by selection for paraquat resistance were found to be long-lived compared to non-selected control lines (135). The lifespan extension of these fly lines was relatively small (~12%) and resistance to other stressors was not tested.

The effects of stress resistance on longevity are also quite inconsistent among several transgenic models for genes in protective and repair systems. For example, the effects of antioxidant overexpression on longevity seem to be dependent on the specific antioxidant enzyme(s) under study, as well as on localization of the over-expressed protein(s). Many early studies had reported conflicting results on lifespan effects of overexpression of CuZn-superoxide dismutase and catalase, over-expressed either alone or in combination (136-138). Some of these early papers were criticized on technical grounds, including uncontrolled background effects, the unexpectedly short lifespan seen in control flies, and the use of native promoters to express transgenes. Subsequent analysis of many of these lines suggested that most of the original transgenic fly lines

were indeed not long-lived (139). More consistent results have come from studies targeting expression to particular tissues, or by use of an inducible promoter like the *FLP*-out system which permit induction of the transgene, in all tissues, specifically in the adult animal. Using the *FLP*-out system, Sun and Tower found that lifespan could be extended about 50% by overexpressing CuZn-superoxide dismutase throughout the adult fly (140). Lifespan effects of targeted overexpression of CuZn-superoxide dismutase are tissue-specific in *Drosophila*; overexpression in motor neurons extends lifespan by about 40%, but expression in skeletal muscle showed no effect (141;142). The effects of this genetic manipulation also are dependent on sex and genetic background (143). Overexpression of Mn-superoxide dismutase extends lifespan in *Drosophila* by about 30% when targeted to motor neurons (142) and by about 70% when induced throughout the body using a *FLP*-out system (144). In contrast to the superoxide dismutases, targeted overexpression of catalase in *Drosophila* motor neurons had no effect on lifespan (142) and induced expression of catalase in the whole body made flies resistant to peroxide, but had little to no effect on lifespan (140). In addition, simultaneous overexpression of catalase with either CuZn- or Mn-superoxide dismutase had no additive effects, while overexpression of both superoxide dismutases did have partially additive effects (140;144;145). Thus, it seems that the activities of the superoxide dismutases have more effect on longevity than does catalase overexpression in this organism.

While the antioxidants represent a resistance mechanism for only a single type of stress, oxidative stress, the heat shock proteins (HSPs) may be important in a general response to many damaging agents for reasons described previously in this chapter (88;89). Several groups have reported that overexpression of different HSPs in *Drosophila* can extend lifespan. Transgenic *Drosophila* expressing multiple copies of *Hsp70* have been shown to live nearly two-fold longer than their controls (146). Overexpression of *Hsp70* also increases survival of flies following heat shock or treatment with paraquat (147). Similarly, overexpression of *Hsp22* in either the whole animal or specifically in the motor neurons extended lifespan by more than 30% relative to control (148). Overexpression of *Hsp22* in motor neurons also rendered flies resistant to paraquat-induced death (148). Surprisingly, a second group has reported that

ubiquitous *Hsp22* overexpression renders flies sensitive to heat and oxidative stress and leads to diminished lifespan (149). Transgenic flies from these two groups differed in their relative levels of *Hsp22* expression, the timing of expression and the system used to induce overexpression, and these differences may have contributed to the disparate findings. It seems then that HSPs may be able to regulate both longevity and stress resistance, perhaps by combating stress- and age-related dysfunction of proteins. This mechanism is in some ways supported by a report that overexpression of protein carboxyl methyltransferase (PCMT), extended longevity about 35% (150). The activity of PCMT repairs isoaspartyl residues that arise spontaneously during the aging of proteins by deamidation of asparaginyl residues, and thus PCMT may extend longevity by its ability to maintain proteins in their native, functional state.

An exciting report by Wang et al. used a novel approach for identification of genes that may regulate longevity by enhancing stress resistance (151). This group generated cDNA libraries enriched for mRNA sequences that were upregulated in flies exposed to one of three stresses (paraquat, heat, or starvation). These libraries were then screened by microarray to identify genes upregulated by each stress, and in particularly, genes upregulated by all three stresses. In the triply-expressed group, they found upregulation of several genes already known to affect longevity, including those that encode catalase, CuZn-superoxide dismutase and HSP-70. Within this group, they also found upregulation of several genes with unknown longevity effects and chose two, *hsp26* and *hsp27*, for further analysis. Overexpression of either of these genes increased resistance to heat and resistance to paraquat and also extended lifespan about 30%. These results thus represent the strongest argument that genes that regulate resistance to multiple forms of stress can also regulate longevity in flies.

Calorie restriction, longevity and stress resistance in *Drosophila*

Calorie restriction has been shown to extend lifespan and delay the rate of aging in most model organisms tested. However, in *Drosophila,* the effects of calorie restriction are often inconsistent depending on details of the restriction regime. These variations in restriction approach reflect in part technical issues in *Drosophila* husbandry.

As is the case for *C. elegans,* it is technically difficult to measure the actual caloric intake of the flies. Further, one group has shown that the lifespan-extending effects of their chosen protocol are not governed by calories per se, but instead by the specific nutritional components of the food (152). Three primary methods of dietary restriction (DR) have been evaluated in flies. The first uses intermittent feeding, in which flies are only allowed access to food for a fraction of the day. DR of *Drosophila* by this method was reported to have no effect on longevity (153). A second method was based on dilution of live yeast in the *Drosophila* rearing media. Typical media for rearing *Drosophila* contain live yeast to keep fecundity high and to promote larval development. Longevity experiments using this method of DR have provided contrasting results: one group has shown that longevity of wild-type flies is unaffected by DR (154), while another suggested that DR extends lifespan to a small degree (155). However, the differences in these experiments might be explained by the different fly strains used in each study.

The method of DR that has produced the most consistent increases in lifespan in *Drosophila* involves dilution of the rearing media itself, with dead yeast present instead of live, growing yeast. This method of DR has led to fairly consistent increases in lifespan across multiple fly strains, for both sexes of flies, and for sterile, virgin and reproductive females (85;156-161). One complication, however, is that this mode of DR causes a drastic reduction in fecundity in *Drosophila,* particularly in females (162). Thus, the increase in survival associated with DR in reproductive females may, in part, be caused by diminishment of fecundity as discussed above. But many of the age-extending effects of this method of DR seem to be independent of female fecundity, because this DR regime can extend longevity in males, in virgin females and in female-sterile fly strains (160). Nonetheless, this issue should be considered in the design and interpretation of *Drosophila* DR experiments.

Most of the sparse information of the mechanisms of delayed *Drosophila* aging by DR has been reported using dilution of media as the means of restriction. The limitied information on the effects of this dietary regime on fly stress resistance suggests that DR has little effect on stress responses. Although DR *Drosophila* are resistant to starvation-induced death (155), they are not resistant to the lethal effects of heat, cold or oxidative stress (152;163). The limited data on stress resistance in Drosophila, combined with the

technical differences in the approaches used for dietary modulation, make it difficult to draw clear conclusions on the question of whether stress resistance may be involved in lifespan extension in this species.

Pharmacological extension of lifespan in *Drosophila*

Several pharmacological interventions have been tested for lifespan effects in *Drosophila*, including several studies of agents that protect cells from oxidative stress. For instance, dietary uptake of N-acetylcysteine (NAC), an antioxidant and precursor to glutathione, extended both median and maximum lifespan in a dose-dependent manner; flies fed low doses of NAC lived about 20% longer than controls and flies fed high doses lived about 30% longer (164). Similarly, dietary treatment with propyl gallate (PGL), an antioxidant with similar activity to superoxide dismutase, has been shown to extend *Drosophila* lifespan (165). Dietary supplementation with PGL for the entire adult *Drosophila* lifespan increases median lifespan by about 30%; treatment for shorter lengths of time resulted in smaller lifespan extension. Unfortunately, direct assessment of stress resistance of NAC- or PGL-treated flies was not reported. However, the effects of dietary supplementation with another antioxidant, melatonin, have been shown to affect both lifespan and stress resistance in *Drosophila*. Flies treated with melatonin have increases in mean and maximal lifespan of 20% and 30%, respectively, relative to control flies (166). Furthermore, melatonin treatment increased the resistance of *Drosophila* to death caused by treatment with paraquat or high temperature (166). In contrast to these findings, dietary treatment with the superoxide dismutase/catalase mimetics EUK-8 and EUK-134 did not affect wild-type *Drosophila* lifespan, nor did it protect these flies from paraquat toxicity (167). This is also in contrast to the (disputed) effects of EUK-8 and EUK-134 in *C. elegans*; antioxidant mimetics were shown to protect worms from paraquat toxicity and, in the experiments of one group, extend worm lifespan (74;77). MitoQ and TPMP, two mitochondria-targeted antioxidants, also had no effect on wild-type *Drosophila* lifespan or paraquat resistance (167). Because of these inconsistencies among the antioxidant treatments, the interpretation of the general effects of oxidative

stress protection on longevity is complicated. In general, it seems that protection from oxidative damage alone is not sufficient to extend lifespan in flies.

The effect of agents that are, in part, independent of oxidative stress have also been tested for their effects on lifespan. 4-phenylbutyrate (PBA) is known to inhibit the activity of histone deacetylases, thus inducing hyperacetylation of histones. The PBA-mediated release of histones from their binding to chromatin is then thought to affect gene transcription (168). Dietary administration of PBA throughout adult life was shown to extend both median and maximum lifespan by about 40% in multiple strains of *Drosophila* (169). PBA also extended lifespan when administered during the first 12 days of adult life alone, and PBA administration did not affect adult size or fecundity of flies. Furthermore, PBA treatment rendered flies resistant to paraquat-induced lethality (169). PBA is not known to have antioxidant activity; therefore, it seems that PBA is able to upregulate mechanisms that affect both longevity and resistance to oxidative stress. Similar effects on longevity have been found using other inhibitors of histone deactylases; treatment with either tricostatin A (TSA) or sodium butyrate (BuA) lengthens fly lifespan by about 25% relative to untreated controls (170). The effects of these treatments on stress resistance was not tested, but both TSA and BuA significantly increased the expression of *Hsp22* and *Hsp70*, suggesting that they may affect the stress response (170). Resveratrol supplementation has also been shown to extend *Drosophila* lifespan about 20% when administered to adult flies, similar to the results shown in *C. elegans* (85;171). Stress resistance of resveratrol-treated flies has not been reported. Overall, these data thus give hints that stress resistance and longevity may be enhanced by pharmacological means, but give little information on the mechanism through which these agents may work.

Biochemistry of long-lived *Drosophila*

Biochemical analyses of anti-aging effects in flies have focused on the activities of antioxidant enzymes and HSPs. The studies of antioxidant enzymes have produced inconsistent results. If resistance to oxidative stress is a primary pathway for longevity extension, it might be predicted that longer-lived animals would resist the effects of

reactive oxygen species by elevation of protective enzymes and metabolites. Some evidence exists that supports this argument. Long-lived *chico* flies have higher levels of CuZn-superoxide dismutase, but Mn-superoxide dismutase is no different between *chico* and control flies; catalase levels have not been reported (125;172). The long-lived "Old" selected lines from Luckinbill tend to have higher mRNA expression of CuZn- and Mn-superoxide dismutase and catalase, and greater activity of CuZn-superoxide (173;174). However, others have generated inbred lines from subpopulations of these flies and found that "Old" and "Young" lines do not differ in the activity of CuZn- or Mn-superoxide dismutase or in total content of glutathione (116). Further, long-lived fly lines selected for paraquat resistance were shown to have relatively low CuZn-superoxide dismutase and catalase activities despite being resistant to paraquat toxicity (135). These data show that long-life does not necessarily correlate with high levels of antioxidant activity, and provide further support for the idea that oxidative stress is unlikely to be a primary regulator of longevity.

The extension of fly lifespan by overexpression of several HSPs, including *Hsp22, Hsp23,* and *Hsp70,* raises the possibility that the activity of molecular chaperones may regulate some aspects of longevity. The endogenous expression levels of several HSPs increase with age including *Hsp22*, *Hsp23* and *Hsp70*, possibly as an adaptation to age-related increases in misfolded proteins (175-177). One might predict that long-lived models may exhibit higher expression of HSPs early in life that may then delay aging phenotypes due to dysfunction of misfolded proteins. The analysis of some mutations and treatments that extend fly lifespan supports this hypothesis; *Hsp22* mRNA expression is higher in the five "Old" selected fly lines generated by Rose (178). Furthermore, TSA and BuA, both of which extend fly lifespan, also increase *Hsp22* and *Hsp70* mRNA expression relative to non-treated flies (170). Hormesis by transient heat treatment has also been shown to induce life-long increases in *Hsp70* expression in addition to longer life (179). *Hsp70* expression is increased in long-lived transgenic JNK signaling flies, as is *Hsp68* (132). Thus, these data support the idea of coordinate regulation of HSPs with longevity, but much more exploration into the regulation of these proteins in other long-lived models is needed.

Extended longevity and stress resistance in mammals

Until recently, calorie restriction has been the only model of extended longevity that has been used to explore the mechanisms that regulate aging in mammals (64). The first report of a single-gene mutation that extended life span in a mammal was that of the Ames dwarf mouse, which has a homozygous recessive mutation in *Prop-1* (Prophet of Pit1). These mice were shown to have a mean lifespan of 49-68% greater than their littermate controls, and a maximum lifespan of 20-50% greater than control, depending on sex (180). Since this first report in 1996, a number of other mutations that render mice long-lived have been discovered or generated. Their utilization has, and will continue, to contribute to our understanding of the mechanisms that regulate the aging process in mammals.

Stress resistance of long-lived mutant mice

Often, the mutations that extend lifespan in mice also render the mice themselves resistant to oxidative damage and death caused by agents that induce oxidative stress. The *Prop-1* mutation in Ames dwarf mice leads to defects in the development of the anterior pituitary and subsequent lack of adult thyroid stimulating hormone, growth hormone, and prolactin, with secondary deficits in IGF-I and thyroid hormones (181). These endocrine changes lead to a maximum lifespan approximately 40% greater than that of their controls and also render Ames dwarf mice resistant to death caused by paraquat, a potent inducer of oxidative stress. After a single intraperitoneal injection of paraquat, Ames dwarf mice survived a significantly longer time than did paraquat-injected control mice (182). Snell dwarf mice have a mutation in the *Pit1* gene, whose gene product acts downstream of *Prop-1* and also regulates the development of cells in the anterior pituitary. Thus, Snell dwarf mice are phenotypically similar to Ames dwarf mice. They too have been shown to live about 40% longer than their controls (183) and, in unpublished reports, also survive for a longer period of time following paraquat injection than do paraquat-treated control mice (A. Bokov and A. Richardson, personal communication). Injection of mice with 3-nitropropionic acid (3-NPA) induces

mitochondrial free radical generation in tissues and phosphorylation of proteins in the MAPK/ERK kinase (MEK) signaling cascade and phosphorylation of c-Jun. After treatment with 3-NPA, phosphorylation of these proteins is lower in the liver of Snell dwarf mice, suggesting that this tissue is protected, in vivo, from oxidative damage (184). In another long-lived model, female mice carrying a heterozygous deletion of the IGF-I receptor (*IGF-IR* +/-) live up to 33% longer than their controls, but there is no significant extension of lifespan in male mice with the same genotype (185). In addition, female *IGF-IR* +/- mice, but not males, live significantly longer following paraquat injection (185). Interpretation of these results requires caution because the mice were reared in a colony that was not specific pathogen free, and the mean longevity of both control (568 days) and mutant mice (756 days) is lower than the mean survival of many other laboratory stocks. Thus, the differences in lifespan, and perhaps resistance to paraquat, may in part reflect differences in response to infectious agents. A similar IGF-I receptor deficiency has also been shown to render mice relatively resistant to death caused by hyperoxia-induced lung damage (186). Mice lacking the gene product of $p66^{shc}$, a downstream effector of IGF-IR, live 30% longer than their controls and survive significantly longer than control following administration of paraquat (187). Further, $p66^{shc}$ knock-out mice show reduced necrosis and apoptosis of muscle and endothelial cells following induction of localized oxidative stress by ischemia and ischemia with reperfusion (188). Finally, the gene product of *Klotho* is thought to inhibit signaling through the insulin/IGF-I signaling pathway, and its overexpression can extend lifespan by up to 30% (189) and render these mice resistant to paraquat-induced death (190).

Of the mutations discussed above, most share genetic alterations that that are thought to inhibit IGF-I signaling in the mouse, including the Ames and Snell dwarf mice, IGF-IR heterozygote mice, and *Klotho* transgenic mice. The involvement of these pathways in the regulation of aging seems conserved across evolution, in that anti-aging effects of modulation of IGF-I-like signals on lifespan are well-described in *C. elegans* and *Drosophila*, as are the effects of this pathway on stress resistance in nematodes (29;191). In the mouse, mutations that inhibit IGF-I signaling can regulate longevity and organismal resistance to oxidative stress (182;185;190). However, another genetic mutation that inhibits IGF-I signaling and extends lifespan does not affect the resistance

of mice to paraquat. Cells in transgenic mice lacking a function growth hormone receptor (GHR-KO) are unable to respond to growth hormone signaling and thus have low levels of IGF-I. GHR-KO mice live up to 55% longer than their controls, depending on sex and genetic background (192;193). Following paraquat injection, female GHR-KO mice died at the same rate as controls and male GHR-KO actually died more rapidly than control (194). Further, GHR-KO mice are sensitive to liver damage caused by acetaminophen, the toxicity of which is thought to be in part due to induced oxidative stress (195). Snell dwarf mice were also found to be sensitive to acetaminophen, in contrast to their resistance to paraquat and 3-NPA (195). In part, the sensitivity of GHR-KO and Snell dwarf mice to acetaminophen might represent differences in conversion to a toxic metabolite rather than resistance to oxidative stress (195). However, the sensitivity of GHR-KO mice to paraquat suggests that long-lived animals need not be resistant to the effects of this oxidative stress. While the regulation of IGF-I signaling obviously can play an integral role in aging, mammalian (and invertebrate) lifespan can also be extended by alterations in pathways that do not directly affect IGF-I signaling pathways (46;48;117;120).

Interestingly, mammalian lifespan might also be extended by transgenic overexpression of genes whose products act to protect cells from oxidative stress. For instance, overexpression of human catalase specifically in mitochondria (MCAT) can extend both maximal and median longevity by about 20% in mice relative to controls (196). However, these researchers were unable to confirm this report on a separate genetic background. MCAT mice were not tested for resistance to stress, but they did exhibit diminished age-related accumulation of oxidative damage to the nucleic acids in skeletal muscle compared to wild-type. The heart-specific overexpression of metallothionein, an antioxidant and metal chelator, can extend the mean lifespan of mice by about 15%, although it does not extend maximum lifespan (197). Because maximum longevity was unchanged, it may be that aging was not altered in these animals, per se. Instead, overexpression of metallothionein in the heart may protect from some common pathology that contributes to death, thereby increasing the average age at which mice die, but not changing the overall aging rate. Again, resistance to stress was not directly tested, but cardiomyocytes from these animals displayed lower levels of apoptosis due to

free radical production. Finally, overexpressing human thioredoxin, which acts as an antioxidant by reducing oxidized proteins, extends mouse mean (35%) and maximum (22%) longevity relative to control (198). However, the mean longevity of both transgenic (~22 months) and control mice (~15 months) was relatively short compared to that which is typically found for laboratory stocks of the same genetic background (C57BL/6). These mice were bred in a specific-pathogen free environment and no data were provided on causes of death, so it is difficult to explain why control mice in this experiment are so much shorter lived than in other laboratories. These mice seemed to be resistant to ischemia-induced oxidative stress in the brain as measured by infarct area following treatment (199).

The three transgenic stress protein models above provide modest support for the idea that lifespan can be extended by upregulation of oxidative stress defenses. However, each report must be evaluated with some caution for reasons described above. In addition, the relative lifespan extension of these transgenic mutant strains tends to be rather modest compared to that insulin/IGF-I mutant mouse strains. This suggests that the aging process is unlikely to be regulated by oxidative stress alone. In support of this idea, long-lived male GHR-KO mice are sensitive to paraquat and female GHR-KO are no different from control (194). Lifespan is extended by about 20% in transgenic mice generated to overexpress α-MUPA (urokinase-type plasminogen activator), a protease whose overexpression is thought to inhibit feeding (200). Like GHR-KO male mice, female α-MUPA mice were found to be sensitive to the effects of paraquat injection; male α-MUPA mice were not tested (201). Also, Snell dwarf and GHR-KO mice are sensitive to the hepatotoxicity of acetaminophen, the toxicity of which may be partly due to oxidative stress (195). These results cast doubt on the hypothesis that longevity is regulated principally by the level of response to oxidative stress. The oxidative stress theory of aging would predict that long-lived mice would be more resistant to agents that induce oxidative stress and conversely, that animals sensitive to oxidative stress should be shorter lived. Mice generated to express reduced levels of Mn-superoxide dismutase (*Sod2* +/-) show high levels of oxidative damage to nucleic acids in many tissues and are sensitive to organismal death by injection of paraquat. However, the lifespan of these mice is no different that that of mice expressing normal levels of *Sod2* (202).

It may well be that the regulation of longevity does not solely affect, or require, resistance to oxidative stress, but that the mechanisms that affect resistance to other types of damaging insults may also be important. For instance, both Snell and Ames dwarf mice exhibit lower incidences of spontaneous cancer (203;204), suggesting that hypopituitary dwarf mice may be protected from the effects of endogenous DNA damage. However, the development of spontaneous neoplasia may be, in part, caused by the development of oxidative lesions in DNA (205). While resistance to oxidative stress is most commonly addressed, some of these long-lived mutants have been assayed for their resistance to other forms of insult. Snell dwarf mice have been shown to be resistant to multiple types of DNA damaging agents that do not cause oxidative damage. In particular, development of skin neoplasia caused by topical application of the chemical carcinogens dimethylbenzanthracene (DMBA) or methylcholanthrene is reduced in Snell dwarf mice compared to controls (206;207). Development of tumors in multiple tissue types following injection with 2-aminofluorene is also reduced in Snell dwarf mice relative to control (208). Following X-ray irradiation of mice, the frequency of mutagenic lesions is significantly lower in the bone marrow cells and spermatogonia of Snell dwarf mice than in control mice (209). In addition, the frequency of mutagenic lesions formed by mitomycin C treatment is significant lower in erythropoietic bone marrow cells from Snell dwarf mice compared to controls (210). In long-lived α-MUPA mice, the incidence of spontaneous tumors, particularly in the lung, is lower than in control mice (211), as is the formation of precancerous lesions and tumors in the colon, stomach and skin following injection with the carcinogens diethylhydrazine or DMBA (201). The brains of at least two long-lived models show resistance to neurodegeneration induced by kainic acid, an excitotoxic agent that acts independently of oxidative stress. Hippocampal neurodegeneration in regions CA1 and CA3 is lower in thioredoxin overexpressing mice following kainic acid treatment (212). A similar result was found in transgenic mice lacking the gene *Surf1*, which encodes a subunit of the mitochondrial respiratory chain. These mice have a mean lifespan about 20% greater than that of their controls; maximum lifespan has not been reported (213). Following kainic acid injection, *Surf1 -/-* mice had diminished neuronal loss in both the hippocampus and the thalamus.

These reports suggest that the mutations that lead to long life also render many of the tissues of these mice resistant to multiple forms of injury.

Taken together, the results presented above suggest that long-lived mutant mice tend to be resistant, in vivo, to the effects of organismal stress. Some models have shown have been shown to be sensitive to oxidative stress, suggesting oxidation resistance alone may not be required for long life. However, the results suggest that resistance to multiple types of stress, including both oxidative and non-oxidative stresses, may be better a indicator of longevity than resistance to a single form of organismal stress alone.

Stress resistance of cells from long-lived mutant mice

The results presented in the previous section represent the bulk of the published reports of stress resistance in long-lived mouse mutants. These reports were made up of in vivo observations dealing primarily with organismal death or accumulation of specific pathologies to particular tissues. In mammals, it may be somewhat naïve to attribute these results solely to stress resistance mechanisms acting at the cellular level. Agents used to induce organismal stress may have unclear modes of action; for instance, paraquat is often described as a systemic oxidative stress, but animal death due to paraquat is most often attributed to lung damage that leads to respiratory arrest (214). In this case, the specific form of tissue injury that leads to the animal's death may be only a distant and indirect surrogate for cellular injury induced by the agent itself. In addition, mammals have many complex regulatory mechanisms by which they buffer the effects of harmful agents, such as evaporative heat loss and blood flow to regulate overheating, or the distribution and activation of inflammatory cells in response to oxidative damage. Further, the effects of stress in mammals are complicated because these animals are made of many vastly different cells that may be differentially protected from the toxic agent due to tissue-specific protective factors, variation in exposure to and accumulation of the test agent, etc. Thus, experimental procedures that expose mice to agents like paraquat provide only limited information about cellular resistance to that agent.

Another approach to address whether long-lived mutant mice are resistant to stress is by using cells derived from these animals to assay their cellular resistance to

stress. Such approaches eliminate many of the complicating factors of assaying organismal stress resistance addressed above. In particular, cell-based assays can provide information about the response to the specific action of a stressing agent rather than the multitude of secondary responses that may contribute to an organism's death. Another important benefit of assays that utilize short- or long-term cell cultures is that multiple experiments could be run in parallel testing the effects of different agents. Such experiments might rapidly determine if long-lived animals are resistant to multiple different agents and in particular to agents whose mechanisms of action are different.

Unfortunately, there are a relatively small number of published reports of stress resistance properties of cells derived from long-lived mammals. In addition, these few reports often only report the results of a single cell type for its resistance to a single agent, or to only a few agents that may induce similar cellular responses. For instance, in the single report on the stress resistance properties of IGF-IR(+/-) mice, it was found that murine embryonic fibroblast (MEF) cultures derived from IGF-IR(+/-) mice are significantly more resistant to cell death induced by hydrogen peroxide relative to those from control mice (185). It was also reported that bone marrow cells from thioredoxin overexpressing mice were significantly more resistant to death by UV irradiation than were analogous cells from control mice (198). At least three cell types from mice lacking *p66*^{shc} have been assayed for stress resistance, which makes these mice the most utilized model for cell-based, in vitro assays of stress resistance by default. MEF cultures derived from *p66*^{shc} knock-out mice are resistant to apoptosis induced by peroxide, UV light, and staurosporine, which non-selectively inhibits protein kinase activity, relative to MEF cultures from control mice (187;215). Thymic and splenic T-cells from *p66*^{shc} knock-out mice are resistant to dexamethasone-induced cell death, although death in untreated cells was lower in *p66*^{shc} knockout mice than in controls (216). Mesangial cells derived from the kidneys of these mice are resistant, relative to control-derived cells, to death caused by incubation in culture media containing high glucose, a rather ill-defined cellular stress (217). These reports on *p66*^{shc} knock-out mice show that this particular mutation confers resistance to many types of stress upon multiple cell types within the mouse. Thus it would be of interest to explore similar types of cellular assessments of stress resistance on other long-lived models.

The rather limited use of cell-based assays of stress resistance in long-lived mutant mice renders this a fertile area for future study. There is obviously much to be learned about whether long-lived animals are resistant to cellular stresses, particularly if they are resistant to multiple forms of cellular stress. However, using cellular approaches to study stress resistance in mammals poses both technical and strategic problems. Evaluation of several cell types, from different tissues, will be needed to clarify the relevance of stress resistance on aging in mammals. In part, the difficulty of cell culture for most cell types from adult animals may limit these choices, as may the growth and survival characteristics of each cell type that can be cultured. Such problems can complicate the evaluation of results from these experiments and complicate interpretation of the role of cell stress resistance in the modulation of the aging process in the animal from which cells were derived.

Stress resistance in dietary restricted rodents

Prior to the discovery that the Ames dwarf mouse was long-lived, the only known means of extending longevity in mammals was by restriction of caloric intake by approximately 1/3 (calorie restriction; CR) (63). For nearly six decades, data have been compiled on the lifespan effects of CR in many animal models (reviewed in (64)). For nearly every physiological, biochemical and pathological measurement, studies using rodents have repeatedly shown that CR decreases the rate of aging and delays the onset of most age-related diseases relative to ad libitum (AL) fed controls (reviewed in (64)). The observation that CR can further extend the lifespan of long-lived Ames dwarf mice (218) and that there are differences in hepatic gene expression profiles between dwarf and CR mice (219) suggest that pathways that extend lifespan by CR are only partly shared with those in genetic mutants. The mechanisms that regulate resistance to stress may be part of those pathways that are shared.

CR rodents (both mice and rats) are similar to many of the long-lived mutant mice in that they have been shown to be resistant to organismal stress. In contrast to the relatively small literature on stress resistance in mutant mice, studies of CR rodents have utilized a great range of stresses, toxins and carcinogens to measure the resistance to

injury of both the animals and of their tissues in vivo. Like many of the long-lived mouse mutants, C57BL/6 mice that have undergone CR are resistant to death due to paraquat injection (220;221). CR of Fisher 344 rats protected these animals from death when they were exposed to high temperature (>33º C for several hours) relative to AL control (222;223). However, CR rats are smaller and this result may thus be due to a greater evaporative heat loss potential rather than heat resistance directly. CR has also been shown to reduce the effects of chronic cadmium toxicity in rats as measured by urinary excretion of proteins and glucose and by loss of bone density over time (224). CR was effective in protecting rats against methylmercury-induced pathological changes in skeletal muscle; small fiber size and suppression of mitochondrial electron transport enzyme activities in skeletal muscle and degenerative changes in peripheral nerves were milder in methylmercury-exposed CR-fed rats than in AL-fed animals (225). CR protects rats from excitatory neurodegeneration in hippocampus caused by kainic acid treatment, and from striatal neurodegeneration caused by 3-NPA and malonate (226). CR also protects rodents from mortality caused by several toxins with differing action including ganciclovir (a hematopoetic toxin), cyclosporin A, phenobarbitol, clofibrate, L-647,318 (an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase), and MK-458 (a dopamine agonist) (227;228). Rats undergoing CR are resistant to liver damage caused by the hepatotoxin thiacetoamide, and the rate of liver regeneration following thiacetoamide treatment is increased with CR (229). The livers of mice that have been treated by CR are protected from the effects of acetaminophen (195). CR-treated rodents also appear to be resistant to many types of DNA damaging insults. The mutation frequency in splenic T-cells is reduced in CR-fed mice subjected to bleomycin, a radiomimetic drug that causes DNA damage through a free radical-dependent mechanism (230). CR has been shown to inhibit the formation of skin tumors in mice and rats following treatment with benzo(a)pyrene (231) or with methylcholanthrene (232) as well as reduce the incidence of mammary tumors caused by DMBA injection (233;234), intestinal tumors caused by methylazoxymethanol acetate injection (235), and myeloid leukemia caused by X-ray irradiation (236). From these data, it is clear that the effects of CR are far-reaching, and that CR provides protection from different sources of insult to many tissues and organs.

While much has been published on the stress and toxin response of CR-treated rodents, there has been relatively little published on the characteristics of cells derived from CR animals. Some studies of CR cellular resistance have measured the ability of cells to repair different types of DNA lesions. For instance, hepatocytes from CR rats repair UV irradiation-induced cyclobutane pyrimidine dimers (CPD) repaired more rapidly in cells from AL animals (237). These assays, which specifically measured nucleotide excision repair, showed that cells in culture could retain the effects of CR even after being removed from the animal. Similarly, a mixture of cell types freshly isolated from the skin of CR rats were protected from UV light and methyl methanesulfonate (MMS) as measured by unscheduled DNA synthesis, a measure of general DNA repair (238). This same measure of DNA repair has shown that CR in rats can increase the rate of DNA repair of UV damage in primary cultures of splenic lymphocytes (239), in secondary fibroblast cultures derived from lung (240) and in cultured liver and kidney cells (241). CR also enhanced the rate of repair of genotoxic lesions caused by the carcinogens 2-acetylaminofluorane, aflatoxin B_1, DMBA, dimethylnitrosamine and tacrine in primary hepatocytes derived from rats (242;243) and of the lesions caused by tacrine in primary hepatocytes from mice (243). In addition to studies of DNA repair, other work has shown that lens epithelial cells isolated from CR mice are resistant to H_2O_2-induced cell death (244), although with extended culturing the resistance of CR-derived cells is lost (245). This particular result might suggest that the effects of CR on cells may be transient in nature. Some data suggest that serum from CR-fed animals can extend "CR-like" phenotypes to cell culture lines, including resistance to cell death induced by peroxide or heat (246;247), but deciding how such experiments relate to the in vivo environment of CR rodents is difficult. Additional studies on how CR influences cellular resistance to stress are clearly needed.

Recently, it has been shown that mammalian lifespan can be extended through another form of dietary restriction. A diet regime restricted in the level of the essential amino acid methionine, provided ad libitum, has been shown to extend lifespan in rats about 30-40% (248;249) and the lifespan of mice by about 10% (250). The limited information on the effects of methionine restriction (MR) suggests that, like CR, MR is able to lengthen lifespan by delaying the rate of aging and progression of age-related

pathologies. In mice, MR retards the development of cataracts and can delay the senescence of the immune system (250). In rats, MR inhibits chronic progressive nephropathy, the major cause of aging-related mortality of F344 rats, and testicular cancer, a common neoplasia in aged rats (251). Even less is known about the stress resistance of these animals, but like CR-treated rodents, mice undergoing MR show less liver damage following acetaminophen treatment (250). MR-fed rodents also may be resistant to chemically-induced cancer; treatment of rats with an MR diet inhibits the formation of colon tumors following azoxymethane treatment (252). There have been no reports on cellular stress resistance of MR rodents prior to the work of this thesis.

Expression of stress defenses in long-lived rodents

Very little is known about the mechanisms that regulate stress resistance among the genetic mutants and rodents on diets that extend longevity. It may be that these animals have increased activity of the proteins that protect from stress-induced tissue damage or animal death. This hypothesis has been primarily addressed by surveying multiple tissues of long-lived and control animals for the expression of proteins that protect from oxidative stress, like that caused by paraquat. In general, there seems to be no clear trend for higher levels of antioxidants in long-lived mice.

Catalase is an antioxidant that protects cells by its enzymatic conversion of peroxide to water and oxygen. As discussed above, MCAT mice overexpress catalase specifically in the mitochondria and live 20% longer than their controls (196). However, the same report showed that targeting overexpression of this enzyme in the peroxisome or nucleus had no effect on lifespan. The activity of catalase has been found to be higher in multiple tissues from Ames dwarf mice, including liver and kidney in two separate reports, and in the heart and hypothalamus in one report each (253-255). Reports on catalase activity in the heart have been inconsistent: the first showed no significant difference, with a trend toward a higher level in control animals (253), but a second group has reported that catalase activity was significantly higher in dwarf animals (254). In contrast to the reports on antioxidant activity in the liver in vivo, the activity of catalase in isolated hepatocytes was lower in cells isolated from Ames dwarf, but because

dwarf samples and control samples were measured in separate experiments, a direct comparison could not be made (256). Catalase activity in the liver of female GHR-KO mice was no different from controls, but in male GHR-KO mice, catalase activity was lower than for their corresponding controls (194). Data on the effects of CR on catalase are even more inconsistent. Mice fed a CR diet seem to express lower levels of catalase mRNA and protein and have lower catalase activity in the liver, eye lens, kidney and skeletal muscle (225;257;258). However, some of these results did suggest that the loss of catalase activity in these tissues with age is attenuated in CR mice (257;258). Others have suggested that mice undergoing CR have higher levels of catalase activity in the liver, brain, heart and kidney (259;260) or that CR attenuates an age-related increase in CR (261). At best, these data suggest that there is no consistent association between long life and catalase activity.

There is also inconsistency in the association of the antioxidant enzymes CuZn-superoxide dismutase (Sod1) and Mn-superoxide dismutase (Sod2) with differences in longevity. These enzymes convert oxygen free radicals to oxygen and peroxide with Sod1 acting primarily in the cytosol and Sod2 primarily in the mitochondria. The activity of Sod1 has been shown to be higher in the hypothalamus (the only tissue tested) of young and middle-aged Ames dwarf, but not in old animals (255). However, the activity of Sod1 is lower in the liver of GHR-KO mice, and there seems to be no difference from control in the kidneys (194). CR also has been shown to have no effect on the expression or activity of Sod1 or Sod2 in liver, kidney, skeletal muscle or eye lens (225;257;258). In contrast, however, there are reports of increases in activity of the Sod proteins in CR models (259;260). GHR-KO mice express higher levels of *Sod2* mRNA in the liver (262), as do *Klotho* transgenic mice in skeletal muscle (190). The expression of *Sod2* in other tissues has not been reported for either model. In contrast, α-MUPA mice showed lower levels of *Sod2* mRNA expression, protein expression and enzyme activity in the liver (201). Additionally, knockout mice with diminished levels of *Sod2* expression have higher levels of oxidative damage and sensitivity to paraquat, but show no decrease in longevity (202). It seems clear from these data that the regulation of superoxide dismutases alone is unlikely to have a major role in regulating longevity.

Finally, the activity of glutathione peroxidase (GpX), which protects mitochondria from oxygen free radicals, also does not correspond well with longevity. GpX activity is lower in the liver and heart of Ames dwarf mice and no different from control in the kidney (263). There is also no difference in the activity of GpX in the liver of α-MUPA mice (201). Liver from α-MUPA mice also showed no difference in the levels of glutathione, a substrate for GpX (211). However, GHR-KO mice showed higher GpX activity than control in the kidney, but no difference in the liver (194). In CR models, GpX has been suggested to be higher, lower and unchanged depending on the tissue, age of the animal, and length of time on CR (225;257-260). Thus, in the published literature there is no clear cut support for the idea that the mechanisms that increase longevity do so by increasing the expression of GpX, or any other antioxidants examined. These data thus make it difficult to support a relationship between oxidative stress defense and lifespan.

There are more consistent data on the expression patterns of proteins that may protect from sources of damage that might be, in part, oxidation-independent, though much less has been published on this topic than on antioxidant defenses. The mRNA encoding metallothionein, an antioxidant and chelator of heavy metals, was shown to be expressed at higher levels in the liver, heart, kidney and brain of Ames dwarf mice and GHR-KO mice (264). Glutathione-s-transferase, which detoxifies many compounds by conjugation with reduced glutathione, is found at higher levels in the kidney of Ames dwarf mice (265), and CR prevents the loss of its activity with age in kidney (266). The heat shock protein HSP-70 is found at higher levels in the Ames dwarf kidney (264), and CR prevents the loss of HSP-27, HSP-72, and HSP-90 with age in the skeletal muscle in rats (267). Methionine sulfoxide reductase, which repairs oxidized methionine residues in proteins, has been shown to be expressed at higher levels in the liver and kidney of Ames dwarf mice (264). These limited data thus suggest that agents that prevent multiple types of damage, rather than merely oxidative damage, may provide be more closely tied to the mechanisms that regulate longevity.

Damage accumulation theories of aging

Oxidation damage, oxidative stress and aging

In 1956, Denham Harman first proposed the free radical theory of aging (268). Prior to this time, it was known that normal cell physiological processes generate reactive oxygen species as by-products and that these free radicals react with many cellular macromolecules, damaging them and producing toxic products. Harman integrated this into his theory which hypothesized that the aging process is a result of the accumulated damage caused by these reactive oxygen species. Under this theory, the aging process is regulated by a balance reflecting the deleterious reactions that produce free radicals, the capabilities of the cell or tissue to protect from these radicals, and the ability of the cell or tissue to repair damaged macromolecules. Since Harman's initial published proposal, one of the primary subfields of biogerontological research has been exploration of the role of oxidative stress in regulation of aging.

For the most part, reports in mammalian models tend to agree that there is an age-related increase in the levels of oxidized lipids, proteins and DNA. For instance, phospholipids in the cellular membranes are particularly sensitive to oxidation by free radicals. The levels of lipid peroxidation products such as malonaldehyde (MDA), 4-hydroxyalkenals (4-HNE) and F_2-isoprostanes are typically substantially higher in samples from old organisms compared to young. In aged mice and rats, higher levels of these lipid peroxides have been shown in the liver (269-271), in kidney (269;270), in lungs (271), skeletal muscle (272), heart muscle (273), and in erythrocytes and blood plasma (271;274). There is also recent evidence that 4-HNE accumulates with age in *C. elegans* and *Drosophila* (275;276). Almost all amino acids are prone to oxidation, which suggests that proteins are a likely a major target of reactive oxygen species in aging animals. Protein carbonyl formation may occur due to direct oxidation of amino acid side chains and by interaction of proteins with oxidative by-products of other molecules such as MDA and 4-HNE. The accumulation of protein carbonyls in the protein pool has been shown to increase in a number of tissues from mammals including the brain of mice, rats and gerbils (277-279), the plasma and lymphocytes of rats (280), the liver of rats

(281), and the hearts of mice (282). Similar results have been found when oxidation of specific proteins has been measured; in the mouse alone there have been reports of age-related increases in oxidation damage of aconitase in the heart and kidney (283;284), β-actin and neurofilamin 66 in the brain (285), BiP/Grp78 and PDI in the liver (286), and ATP-synthase in the heart and skeletal muscle (283). Removal of damaged proteins is mainly achieved by proteolytic degradation pathways including proteasome proteases, lysosome proteases, and mitochondrial proteases. Age-associated impairment has generally been reported in the function of all these proteolytic pathways (287). The primary by-product of oxidative damage to nucleic acids is the formation of 8-oxo-2-deoxyguanosine (oxo8dG) lesions. These lesions have been shown to be mutagenic in mammalian cells and are primarily removed by base excision repair (288). Earlier studies of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have reported inconsistent observations about the accumulation of oxo8dG with age. The first report on the effects of aging on DNA oxidation reported a significant increase in the oxo8dG levels with age in nDNA from rat liver, kidney and intestine but not in brain or testes (289). Others followed with reports that showed a general trend in mouse and rat tissues for the accumulation of oxo8dG with age in both nDNA and mtDNA (290-292), although one report found no difference between young and old (293). At least part of these contradictory results might be explained by oxidation of DNA induced by the DNA phenol extraction itself (294;295). A comprehensive study of two strains of mice and one strain of rats using an alternate method of DNA isolation that does not have this effect showed that the nuclear and mitochondrial DNA had higher levels of oxo8dG in liver, heart, brain, kidney, skeletal muscle, and spleen of aged rodents compared to young (296). Other studies have also confirmed the increase of oxo8dG in mitochondrial DNA from rat liver (297).

If longevity is regulated by the accumulation of oxidation damage, it would be predicted that experimental manipulations that increase longevity by delaying aging rate should reduce the accumulation of oxidatively damaged macromolecules and accompanying tissue dysfunction. Most of the experimental support for this prediction comes from studies on CR treatment of rodents. In general, most reports have suggested that CR reduces the age-related accumulation of oxidized macromolecules. For instance,

the accumulation of lipid peroxidation is lower in CR rodents relative to AL; MDA is lower in liver samples from CR mice and rats (298-301) and CR reduces the levels of F_2-isoprostanes in liver, kidney and plasma (270). Multiple reports also show that CR prevents the age-related increase in levels of oxidized proteins in rodents. For instance, CR reduces the accumulation of protein carbonyls in skeletal muscle mitochondria (302) and in homogenates of brain, kidney and heart (303;304) from mice, and in liver and brain homogenates from rats (305;306). CR also reduced the levels of oxo8dG lesions in DNA from skeletal muscle, brain, heart, liver and kidney of mice, although the interpretation of these results is complicated by the authors' use of an oxidizing DNA extraction process (304). A subsequent report utilizing a non-oxidizing DNA extraction process confirmed these results in nDNA isolated from liver, heart, brain, kidney, skeletal muscle, and spleen and in mtDNA isolated from liver (296). Much less data has been published on oxidative damage reduction in long-lived mutant mice and invertebrates. The resistance to oxidative stress of many of these long-lived mutants and their biochemistry are discussed elsewhere in this chapter. Of the mouse mutants, differences in oxidation damage have only been addressed in the Ames dwarf and *p66*^{Shc} knockout mice. In old Ames dwarf mice, protein carbonyl levels in the liver are significantly lower than in age-matched control mice, but there was no difference in the brain (307). In contrast to CR models however, lipid peroxidation measured by MDA and 4-HNE levels were higher in liver and brain from old dwarf mice compared to control (307). Differences in oxo8dG content between Ames dwarf and control were also inconsistent; Ames dwarf liver had higher levels of oxo8dG in nDNA, but lower levels in brain nDNA and mtDNA, and heart mtDNA (307;308). In older *p66*^{Shc} knockout mice, plasma F_2-isoprostane levels are reduced compared to control (309) and oxo8dG levels were lower in lung, spleen, liver, kidney, and skin but no different in heart, muscle or brain (310). From these data, CR can not only extend lifespan, but also cause reductions in the accumulation of macromolecular oxidation in many tissue types. However, genetic mutant mice show a much less clear pattern of reduction of oxidation, suggesting that it is unlikely that these mice are long-lived simply because they accumulate less oxidative damage.

The free radical theory of aging also predicts that experimental treatments that diminish oxidative stress and the accumulation of oxidation damage should increase longevity. This prediction is perhaps the most interesting of the three because positive results would suggest a causal link between oxidative damage and aging. Tests of this prediction have been inconclusive, with the bulk of the evidence tending to refute the prediction. Most of these results have been presented above in this thesis and their results will only be briefly reiterated. In brief, treatment of *C. elegans* with antioxidant mimetics was shown to extend lifespan by one group and have no effect on lifespan by another group (74;76;77). Both groups found that this treatment rendered worms relatively resistant to oxidative stress, but there have been no reports on the efficacy of this treatment in reducing oxidative damage with age. In *Drosophila*, transgenic techniques have shown that overexpression of CuZn- or Mn-superoxide dismutase can extend lifespan whether expressed in the whole adult fly or specifically in the motorneurons (140-142;144;145). However, overexpression of catalase had no effect on lifespan (140). Again, there is a suggestion that these transgenic flies are resistant to oxidative stress, but effects on oxidation of macromolecules have not been addressed (141). In mammals, lifespan extension has been reported for transgenic mice with mitochondrial-specific overexpression of catalase (MCAT), heart-specific overexpression of metallothionein, and overexpression of thioredoxin (196-198). Reservations about these findings have been previously discussed in this chapter. MCAT mice have been the only model for which an alteration in macromolecule oxidation has been reported; in old MCAT mice, there was a reduction in the accumulation of oxo8dG in the nDNA from skeletal muscle but not from the heart (196). With these data sets, it is difficult to find strong support for the prediction that protection from oxidative stress can extend life. Additionally, dietary treatment of mice with antioxidants seems to be able to diminish oxidative stress and the accumulation of some oxidized macromolecules, but in no report was lifespan affected (311-313).

It is clear that aging leads to accumulation of oxidative damage to macromolecules. Further, some interventions that extend longevity seem to diminish this process fairly consistently, though for others the data are more complicated. However, interventions that reduce oxidative damage do not universally extend lifespan, and there

are serious problems with interpretation of many reports on antioxidant interventions. Thus, it seems unlikely that longevity is solely regulated by the accumulation of oxidative damage, particularly in mammalian models. This does not preclude a partial role for oxidative damage in aging; it may be that the mechanisms of oxidative stress resistance may be regulated in parallel with those that extend longevity.

DNA damage, mutation accumulation and aging

Cellular DNA is under constant challenge by exogenous and endogenous sources of genotoxic stresses that can cause both transient and accumulated damage and genome instability. Damaged DNA may be particularly deleterious because it may lead to cellular death or may become a permanent mutation passed among dividing cells that may eventually lead to aberrant cell function. It may be important to distinguish the effects of DNA damage per se from the effects of DNA mutations because they may each affect aging processes independently. DNA damage induced by agents such as reactive oxygen species or UV light generates chemical alterations to the structure of DNA that are, usually, readily removed by DNA repair processes. When DNA damage is high, cellular responses can be activated that lead to cell senescence or cell death; senescent or dead cells might then contribute to age-related tissue pathologies or dysfunction. Additionally, the accumulation of DNA damage in stem cells might lead to an exhaustion of these cells and contribute to an increasing abundance of senescent cells within differentiated tissues. In contrast, mutations are alterations in the DNA sequence organization that are passed to daughter cells through cell division. Their accumulation within genes, or regulatory regions of genes, may cause the loss of function, or altered function, of the corresponding gene products, which may in turn contribute to the aging phenotype. The maintenance of the genome thus seems to be quite important in the prevention of organismal pathologies associated with the accumulation of DNA damage and mutations. Processes that improve DNA repair, or diminish DNA damage and mutations, might well extend longevity.

A number of reports have suggested that as organisms age, their genome accumulates multiple types of damage. Most of this work has focused on the

accumulation of oxidative damage which was described above. The accumulation of other types of genomic lesion has also been addressed experimentally. Double stranded breaks (DSBs) in nDNA have also been reported to accumulate during the aging process; in samples taken from mouse liver, testes, lung, brain and kidney the foci frequency of the phosphorylated form of histone γ-H2AX, which localizes to chromatin surrounding DSBs, increased with age (314). The frequency of genome alterations and mutations has also been reported to increase with the age of the organism. One of the first such reports showed that following a partial hepatectomy, regenerating hepatic cells from old mice had significantly more chromosomal aberrations than did hepatic cells from young mice (315). However, it is unclear if the method of hepatectomy (carbon tetrachloride injection), induces damage equally in young and old mice. Similarly, the number of mouse peripheral blood lymphocytes and bone marrow cells with genome translocations is significantly higher in samples from old mice compared to young mice (316). Mutation frequency within specific genes has also been suggested to increase with age. By using a selection for loss of hypoxanthine phosphoribosyl transferase (*HPRT*) gene activity, researchers have shown that mutation frequencies increase with age in T-cells from both humans and mice (317;318). However, others have reported that there may be tissue specific differences in the accumulation of mutations with age. These reports have utilized transgenic mouse models expressing chromosome-integrated reporter genes which can measure the frequency of a wide range of somatic mutations in all tissues. These reports have found that mutation frequency increases with age in spleen, liver, heart and small intestine, but not in brain or testes (319-321). A second group confirmed these results using a transgenic mouse with a separate integrated reported gene (322). Both groups have suggested that differences in DNA damage between tissues might regulate these differences in mutation frequency. Unfortunately, there is no evidence directly linking either increased DNA damage or somatic mutations with any age-related pathologies other than neoplasia.

If the accumulation of DNA damage and somatic mutations could regulate the aging rate, it would be predicted that long-lived animals should accumulate both at a slower rate relative to short-lived animals. Relatively little has been done to address this prediction. As discussed in the previous section, CR is known to diminish the

accumulation of oxo8dG lesions with age in multiple organs in mice and rats (296). The frequency of oxo8dG lesions may also be lower in old Ames dwarf, *p66*^{Shc} knockout mice and MCAT mice relative to control, though this seems to be dependent on tissue type (196;307;308;310). At least two groups have found that CR also decreases the age-associated accumulation of mutations in the *HPRT* locus in T-cells; one group found that CR decreased spontaneous mutation and a second group found that CR diminished the rate of mutations induced by bleomycin (230;323). It might also be predicted that rate of DNA repair may be increased in long-lived models. The enhanced DNA repair capability found in multiple tissues from CR rodents was discussed previously (238-243). In addition, DNA repair has been found to be correlated with maximum lifespan across the mammals; this will be discussed in a later section of this chapter. Although there are only limited data, and results appear to differ across tissues, in general long-lived animals tend to have lower levels of DNA damage and lower mutation rates, which may in turn be due to increased ability to repair damage. There is, however, no clear correlation between DNA damage or mutation and pathological dysfunction, except neoplasia.

The clearest support for the role of DNA damage and mutation in regulation of aging would come from experiments in which increased longevity results from interventions that reduce mutations. However, very little has been reported on the use of transgenic models expressing DNA repair components at higher than normal levels. These reports, using either cells or whole animals, have generated conflicting results on whether increases in DNA repair enzymes alter the ability to repair DNA damage. Depending on the protein and DNA repair system affected, such transgenics have been reported to enhance, leave unaffected, or lower DNA repair (324-326). Only two groups have formally reported the longevity of their models, and neither provided strong support for the hypothesis that improved DNA repair would delay aging. In *Drosophila*, overexpression of *mei-41*, a checkpoint protein that is thought to sense DNA damage and regulate cell cycle progression, was shown to have a very small, but significant, increase in lifespan, and deletion of the gene shortened lifespan (327). Unfortunately, this group did not report levels of DNA damage in either transgenic or control flie. In the mouse, expression of the human O^6-methylguanine-DNA methyltransferase, which repairs O^6-methylguanine lesions, lowered mutation frequency in the liver and the diminished the

rate of hepatocarcinoma but had no effect on median or maximum lifespan (328;329). Thus, so far there are few unambiguous conclusions from experiments that have tried to increase the expression of DNA repair proteins. One issue may be that many of these proteins are required for developmental processes, and so altering their expression may have detrimental affects on viability. It would be of interest for future work to bypass this issue by using conditional mutations to generate tissue- and temporal-specific transgenic models of DNA repair.

Many deficits of DNA repair enzymes have been reported and these mutations generally make mice prone to DNA damage, mutation and cancer (330). These mice sometimes develop phenotypes that have been termed "segmental progeria," argued to be a form of accelerated aging (330;331). Although the matter is controversial, the "aging" effects of these mutations are more likely to be caused by animal illness and probably share little with actual aging processes (332). This skepticism is supported by a recent report that mutation rate of the shortest lived "accelerated aging" models are no different from other, longer-lived DNA repair deletion mutants (333).

Overall, these data do not provide much support for the idea that accumulation of DNA damage or mutations can regulate the aging process. Most of the available data are merely correlative, and none of the evidence provides a strong link between the accumulation of damage and the progression of age-related phenotypes. However, the accumulation of DNA damage with age may give us hints about what role genotoxic stress resistance could play in the regulation of longevity. CR and genetic mutations were shown to diminish the accumulation of DNA damage. CR can increase the ability of many cell types to repair DNA damage, and DNA repair rates are correlated with maximum longevity. Thus, it may be that the mechanisms that regulate longevity may be shared with those that regulategenotoxic stress resistance, such as to UV light.

Resistance to multiple forms of damage

In both theories discussed above, there is strong evidence that particular forms of macromolecular damage tend to accumulate with age. However, both theories suffer in that there is relatively little support that the aging rate can be slowed by diminishing the

amount of damage that occurs. Thus, it seems unlikely that the resistance to a single type of stress, be it oxidative or genotoxic, or that the accumulation of a particular type of damage can solely regulate the aging process.

It may be that regulation of the aging process is more dependent on pathways that regulate resistance to multiple forms of stress, rather than to a single type, and that this may a better indicator of longevity than either of the previously discussed theories. As discussed in previous sections of this chapter, the mutations that extend longevity in invertebrates often render these animals resistant to multiple forms of lethal agents. For instance, the *age-1* mutation that extends life span in the nematode worm *C. elegans* also renders the worms resistant to multiple forms of lethal injury, including heat, heavy metals, UV irradiation, and oxidizing agents including hydrogen peroxide and the free radical generator paraquat (20-25). Similar results were found for other long-lived worm mutants as well including *daf-2*, *spe-26*, *sgk-1*, *pdk-1*, *sir-2* and *old-1* mutant worms. Similarly, long-lived *mth* mutant flies are resistant to both heat and the oxidative stressor paraquat (117), as are flies in which the cells that produce Drosophila insulin-like peptides have been ablated (128). There is also a growing pool of evidence that interventions that extend lifespan in mammals, particularly CR, can confer resistance to multiple forms of stress including oxidative stress, heat, and a variety of different toxins and mutagens (220-230).

These observations have suggested that the mutations may prolong life span because they render some or all cells within the worm or fly, and perhaps even in vertebrates, resistant to various forms of damage from intrinsic or extrinsic agents. It may be that resistance to these multiple agents then provides protection to the cells, tissues and organs from the accumulation of many types of damage over time, including oxidation, DNA damage and breaks, and mutation among others. Thus, this increased cellular maintenance might then directly contribute to delaying the occurrence of age-related pathologies and slowing the rate of aging. Further, evolution of longer lifespans among groups of different species might proceed by alteration of these intrinsic stress defense pathways, thereby protecting cells for their newly-evolved prolonged periods of life.

The strongest support for this theory, like the damage theories discussed previously, would come through experiments that show extension of lifespan by increasing resistance to multiple forms of stress. As discussed in other sections of this chapter, increasing resistance to a single stressor, primarily oxidative stress, yields inconsistent results on lifespan depending on a number of factors including the model, the target, and the methods used. However, methods wherein pathways that regulate resistance to multiple forms of stress are altered have yielded more consistent lifespan extending effects. For instance, overexpression of heat shock proteins in *C. elegans*, or pathways that regulate heat shock proteins (HSPs), confer resistance to multiple forms of stress, heat and oxidative, and extend longevity (91-93;97). In addition, using a GFP-reporter system, it was shown that individual worms with high HSP response lived longer than worms with lower response to stress (94;95). Similarly, overexpression of HSPs has also been shown to increase lifespan in *Drosophila* (146-148). These data also include the work of Wang et al. (151) who generated long-lived transgenic HSP fly lines based on the results of a selection for genes that conferred resistance to three forms of stress. All of these findings have been discussed in more detail elsewhere in this chapter.

Thus, it may be that longevity is regulated by protection from multiple forms of extrinsic and intrinsic stressors. As an extension of this idea, we might then better understand the mechanisms of aging by exploring how multiple cellular stress resistance is regulated.

The Snell dwarf: a mouse model of extended longevity

Animal models of altered longevity and aging rate provide valuable tools in the study of the regulatory mechanisms of the aging process. In particular, models of extended longevity provide researchers with a tool that can be used to address specific scientific questions about what may be necessary for long lifespan. In the past few years, a number of invertebrate models have been developed utilizing the power of genetics in *C. elegans* and *D. melanogaster* (334-337). The findings that single gene mutations could lead to extended lifespan and a delayed rate of aging in these models has led others to develop mammalian, particularly mouse, models of extended longevity.

CR has long been known to extend the lifespan of mice, as well as other animal species, and much insight into the aging process has been gained by studies of CR rodents (64). However, the mechanisms by which calorie restriction exerts its effects are not well-defined and are likely to involve multiple biochemical and physiological pathways. In contrast, single gene mutations can be used to define how aging can be modulated by a specific protein or pathway and thus can be used to address specific hypothesis about the mechanisms that regulate the aging process. In addition, once it is known that these mutations extend lifespan, these mouse strains can be examined in early life to provide information on mechanisms that differ from control mice and may delay the aging process. As discussed above, the use of mutant mouse strains to address specific biogerontological hypotheses has become a major theme in aging research in the past decade (180;183;185;187;189;192;200;338).

My dissertation research focuses on the Snell dwarf mutant mouse. The first report of these mice, by G.D. Snell in 1929, showed that the mutant was recessive, and that homozygotes were healthy, but sterile and less vigorous than controls, with an adult size approximately 1/4 that of control mice (339). The original Snell dwarf mutation was later found to be a point mutation at the *Pit1* locus (pituitary-specific transcription factor 1) (340). The mutation interferes with the action of its gene product Pit1, a trans-acting POU domain protein necessary for the differentiation of pituitary somatotrophs, thyrotrophs and lactotrophs, the cells responsible for secretion of growth hormone, thyroid hormone and prolactin, respectively (340;341). Therefore, Snell dwarf mice are deficient for these hormones, as well as their secondary effectors, shortly after birth. These alterations in the hormonal profile cause the growth retardation and diminished adult size of these mice that was first reported by Snell. Most reports now find that the adult size is approximately 1/3 that of control mice (183;342).

The hormonal alterations that accompany the Snell dwarf mutation also make these mice long-lived compared to their littermate controls. Initial reports of the lifespan of the Snell dwarf mouse suggested that this mutant strain was rather short-lived (3 – 5.5 months) and thus represented a potential model of accelerated aging, particularly of the immune system (343). However, even control mice were relatively short lived in this study (maximum lifespan ~2 years) suggesting that poor housing conditions probably

contributed to the diminished lifespan of the dwarf animals. Further, many of the parameters of aging other than lifespan that were used in this study, such as hair graying and loss, atrophy, and in vivo cellular turnover are more indicative of illness rather than aging. Others reported that a coallelic dwarf strain, the Jackson dwarf mice (*Pit1*$^{dw\text{-}J}$) had no lifespan reduction compared to control and hinted that these mice may actually outlive their littermate controls (344). This strain has a separate mutation in the *Pit1* locus than the Snell dwarf, but the two mutations have similar effects on the differentiation of the pituitary cells and an indistinguishable phenotype. This study suffered due to small sample sizes and a lack of statistical analysis of lifespan data. Therefore, an independent lifespan study was performed, which found that coallelic hybrid Snell dwarf mice carrying (*Pit1*^{dw}/*Pit1*$^{dw\text{-}J}$) have a mean lifespan >40% greater than that of their littermate controls (Snell dwarf mean 1,178 ± 235 days vs. control mean 832 ± 158 days) when housed in specific-pathogen free conditions and with a control littermate to provide warmth to the hypothermic dwarf mice (183). In addition to mean lifespan, it was found that maximum longevity was approximately 37% greater in Snell dwarf mice (Snell dwarf 1,451 days vs. control 1060 days). Further, analysis of mortality curves suggested that the effects of aging on lethal illness in dwarf animals are delayed by about 12 months, but that once initiated these effects increase at approximately the same rate in each genotype. These results were replicated by a study of the Snell dwarf mutation on a second mouse strain (345), suggesting that the mutation and its effects can extend lifespan regardless of genetic background. These results are very similar to those found for a separate hypopituitary dwarf mouse mutant, the Ames dwarf mouse. Ames dwarf mice were first described as a recessive mutation leading to dwarfism in 1961 (346), and later shown to reflect a point mutation in the gene *Prop-1*, an upstream activator of *Pit1* (347). Because the mutations are in the same pituitary development pathway, the Ames dwarf and Snell dwarf mice share most of the same endocrinological phenotypes. Ames dwarf mice were found to have a mean lifespan of 49-68% greater than their littermate controls, and a maximum lifespan of 20-50% greater than control, depending on sex (180).

Not only are both of these hypopituitary dwarf models are long-lived, but evidence also suggests that this longevity is accompanied by delay or diminution of many

late-life associated pathologies. Because of their similar genetic mutations and the resulting similar patterns of alterations in the endocrine system, the delayed and diminished pathologies of Ames and Snell dwarf mice will be discussed in parallel. Snell dwarf mice show a decline in the rate at which collagen cross-links occur during aging (183). The proportion of CD4 and CD8 T-cells that express markers of memory and P-glycoprotein activity is significantly lower in old Snell dwarf mice, indicative of a lower degree of immunosenescence than that of control mice (183). Age-dependent pathology of the knee joints and vertebral column is greatly retarded in Snell dwarf mice and age-related osteoarthritis is completely prevented (348). The incidence of cataract formation with age is greatly reduced in Snell dwarf mice, as is the severity of glomerular basement membrane damage in the kidneys (203). Tests of mental acuity and measures of locomotion show that Ames dwarf mice have a delay in the rate of age-related cognitive decline (349;350), perhaps because of a higher level of neurogenesis in the brain (351). Both Snell and Ames dwarf mice have been shown to have lower incidence and presumably slower progression of multiple forms of spontaneous neoplasia (203;204). Further, Snell dwarf mice are resistant to the development of neoplasia in multiple tissues when induced by at least three different chemical mutagens (206-208). Cancer progression may be inhibited by means of DNA damage protection; the frequency of X-ray- or mitomycin C- induced mutagenic lesions are significantly lower in Snell dwarf bone marrow cells and spermatagonia relative to controls (209;210).

Ames dwarf mice have also been shown to display increased expression of proteins that influence resistance to stress, in particular those associated with resistance to oxidative stress. The published literature on this point is not entirely consistent, but most reports suggest that proteins that protect from many types of damage are more active in tissues from long-lived dwarf mice. The enhancement, or lack thereof, of these proteins was discussed in a previous section of this chapter. The data show that cellular defenses to stress, primarily oxidative stress, are upregulated in many tissues from the Ames dwarf and predict that these animals, and their tissues, may be resistant to oxidative damage. There is limited experimental evidence supporting this prediction in whole animal models. These results too were discussed in a previous section. In brief, Ames and Snell dwarf mice are relatively resistant to death caused by paraquat (182)(A. Bokov and A.

Richardson, personal communication). Further, responses in the MEK-ERK kinase signaling cascade and in the phosphorylation of c-Jun are reduced in the liver of Snell dwarf mice after treatment with 3-NPA, which induces mitochondrial free radical generation (184). In addition, tissues from the Ames dwarf mice tend to exhibit lower levels of oxidative damage with increasing age as measured by inorganic peroxides in the liver and kidney (352;353), protein carbonyls in the liver and brain (308), and oxidative damage to the mitochondrial DNA in the heart and brain (307). This suggests that the changes that lengthen life in Snell dwarf mice can also confer oxidative stress resistance to particular tissues in vivo.

These data, and the previous work in invertebrate models, give hints that long-lived Snell dwarf mice might be resistant to multiple forms of stress. For reasons stated in a previous chapter, it is difficult to interpret the in vivo resistance of whole mice to agents such as paraquat. The limited data from other models of longevity suggest that some cells isolated from long-lived mice may retain, in culture, stress resistant characteristics. The use of cells would also provide a convenient model in which molecular and cellular biology techniques could be used to delineate the molecular mechanisms of stress resistance, and perhaps give clues to the mechanisms by which lifespan is regulated. Thus, in this thesis we wished to test the hypothesis that the changes that make Snell dwarf mice long-lived also render their cells relatively resistant to the effects of multiple agents that induce cell stress. Further, by developing cell-based assays of stress resistance we could then test the hypothesis that the mechanisms that regulate cellular stress resistance are shared amongst multiple models of extended longevity.

Several pieces of evidence suggest that the genome of Snell dwarf mice may be relatively protected from damage compared to control mice. These data include the relatively low incidence of spontaneous cancer development in Snell dwarfs (203), their resistance to development of tumors following chemical carcinogenesis (206-208), their lower levels of mutagenic lesions following DNA damage (209;210) and lower levels of oxidative damage to DNA (307). However, nothing has been reported on the mechanisms by which DNA damage is kept at lower levels in these mice. Therefore, we

wished to address, in a cell culture model, why Snell dwarf mice may be protected from specific types of DNA damage.

Comparative biology of aging

Rationale for using comparative biology to study longevity

Single gene mutations do not extend lifespan by more than 75% in any rodent, with approximately 50% extensions typical for most studies (354). Within the class of mammals, species have evolved maximum lifespans that differ by more than 100 years, and even within a single order the longest-lived species of rodents can in some cases live more than 10-fold longer than the shortest-lived (2;355;356). The mechanisms that produce such vast differences in longevity among closely related species are almost entirely unknown. In particular, it is unknown if the evolution of longevity always proceeds through the same pathways, or if mechanisms are unique to each particular phylogenetic branch or even species. If, throughout evolution, mechanisms that regulate extended longevity also affect the mechanisms of resistance to multiple forms of stress, we might thus predict that cells from long-lived species should also be resistant to stress. As an extension of this prediction, the delineation of the pathways that regulate cellular stress resistance across mammalian species may thus provide us many clues on the means by which Nature creates long-lived animals.

Pitfalls to comparative biology approach

Experimental designs that compare animals of several species face several logistic and technical obstacles. Information on the physiological, biochemical and particularly cellular processes in informative species is often unavailable or less reliable than information about animals traditionally used for agriculture or in laboratory studies. This is particularly evident in the field of gerontology, where tabulated data on maximum lifespan is too often based on a single colloquial report or a small number of poorly documented observations. Interpretation of lifespan data can also be complicated by

ecological or physiological characteristics of particular species, or groups of species, that are independent of longevity. For instance, some birds and terrestrial mammals may have similar lifespans, but the metabolic demands of flight may affect cellular physiologically in drastically different ways. Designing experiments to address such complications is a difficult task. It is also possible that clusters of related species may share inherited adaptations that influence lifespan; regression analyses that treat each of these related species as an independent observation may be misleading unless adjusted to weight observations based on degree of phylogenetic independence.

The well-known relationship between body size and lifespan across species - with larger species often showing slower aging and longer lifespan than smaller species - also complicates design and interpretation of comparative analyses of aging. Commonly, maximal lifespan is used a measure of longevity and aging. Among vertebrates, there tends to be a correlation between maximum lifespan and body size, with long-lived animals tending to be much larger than short-lived animals (2;357). There are many exceptions to this correlation; bats, for instance, live much longer than expected for their body size (357) and birds, when compared to mammals, live longer than expected for their body size (358).

Because of this relationship, a particular trait that shows a correlation with lifespan is also likely to have a correlation with body size. For instance, basal metabolic rate tends to be correlated positively with lifespan and negatively with body size. Many physiological traits vary with metabolic rate, including many that have been hypothesized to regulate the rate of aging, such as free radical production and, indirectly, the accumulation of oxidized cellular macromolecules. Thus, if we were to measure the accumulation of oxidized proteins across mammals with differing longevity, we may find that long-lived animals tend to accumulate less damage. It is unclear if correlations of this kind reflect the mechanisms that regulate aging rate, or those that regulate metabolic rate, itself strongly correlated to species-specific lifespan. It may be that the two are not entirely independent, but the confounding factor of body size must be addressed by statistical strategies that can remove the body weight portion of the association. Such strategies tend to assign each species a normalization factor based on the relationship between lifespan and size for a particular species. An example of such a factor is the

calculation of a "longevity quotient," a ratio of the species's maximal life span to the average life span of mammals of the same size. Subsequent analyses of the characterization of interest can relate this trait to longevity quotient rather than maximum species longevity (357). One problem with such strategies is that this adjustment may actually obscure the analysis of traits that do modulate aging rate, and do vary with body size. Thus, evidence that stress resistance pathways vary among species might in principle reflect an artifact of association with body size or metabolic rate, or might reflect an important element in the evolution of slower aging. Data from correlative studies of the comparative biology of aging must then be interpreted with this in mind, and ideas inferred from comparative analyses about the cellular and molecular factors that regulate aging rate will require evaluation using alternate experimental approaches.

Comparative biology of aging and stress resistance

A number of published reports have explored the role of oxidative stress and longevity across a number of mammalian species. Early reports from several groups have tried to make the case for antioxidant activity as a regulator of longevity. However, there is much inconsistency among these reports and some of the studies have ignored the pitfalls of comparative biology discussed above and thus pose problems in interpretation. For instance, an early report suggested that the activity of CuZn-superoxide dismutase (Sod1) was positively correlated with maximum lifespan in samples taken from liver, brain, and heart samples of 2 rodent and 12 primate species with lifespans ranging from 3.5 to 95 years (359). However, in this study Sod1 activity was normalized by the basal metabolic rate of the animal from which samples were collected; there was no relationship between non-normalized enzyme activity and maximum longevity. Since the animals used in this study greatly differed in body size, and larger mammals have low metabolic rates, it is clear that the correlations reported may well reflect differences in metabolism rather than in longevity. The results from several other studies have suggested that across mammalian species, the activities of Sod1, catalase, and glutathione peroxidase (GpX) are actually negatively correlated with maximum longevity (reviewed in (360)); that is, long-lived animals tended to have lower activities of these enzymes.

These results were compiled from studies using tissue samples taken from kidney, liver, or brain from at least 5 different mammalian species in each study. The most compelling evidence for a positive correlation between longevity and antioxidants comes from a report that measured Sod1, catalase and GpX in brain, kidney and liver from 6 different mammalian species (with lifespans from 3.5 to 30 years). In this study, the investigators found positive correlations between antioxidant activity and longevity for Sod1 in all three tissues, catalase in kidney, and GpX in brain (361). In contrast, GpX was negatively correlated with longevity in kidney and liver and no correlation was found for catalase in liver and brain. The inconsistencies in these reports as a group may have multiple sources; not all reports tested the same tissues, nor did they sample from the same species. Partly for these reasons, there was no obvious relationship between the overall level of antioxidant defenses and lifespan among the mammalian species and tissues examined.

Several groups have attempted to directly explore the oxidative resistance of tissues or cells from species of differing lifespan. In contrast to inconsistencies in the levels of antioxidants, reports on stress resistance across mammalian species tend to show that cells from long-lived species are resistant to oxidative stress. For instance, one group reported that oxidative stress resistance of brain and heart homogenates from five different species (mouse, rat, rabbit, pig, and pigeon) was positively correlated with lifespan (362). In this study, homogenates were measured for the formation of protein carbonyls following irradiation with X-rays, which cause oxidative stress by generating superoxide radicals from the breakdown of water. This report needs to be interpreted with caution, however, because of the decision to use tissue homogenates, each of which represents a collection of many types of cells. A further complication is that this report compares samples prepared from whole organs of small animals to specific regions of organs in the larger animals. Another set of studies has reported that cells derived from birds are more resistant to oxidative stress than cells from mammals (363;364). In the first report, these researchers found that renal epithelial cells derived from budgerigars, European starlings and canaries (all with captive longevities >20 years) were more resistant than cells from lab mice to cell death induced by oxidative stress generated by hyperoxia, paraquat, or γ-irradiation (363). The second study expanded on these results

and found that embryonic fibroblasts from budgerigars and Japanese quail (lifespan ~ 5 years) were more resistant to hyperoxia and hydrogen peroxide than were embryonic fibroblasts from mice (364). However, they also found that bird-derived cells were more resistant to these agents than human embryonic fibroblasts. It may be that this particular result represents the different physiological demands between mammals and bird discussed above in the pitfalls of comparative biology. Thus, such experimental designs may require incorporating statistical methods that account for phylogenetic relatedness, methods that were not used in these assays.

Perhaps the most comprehensive analysis of stress resistance across species was reported from a group that utilized skin-derived fibroblast cultures derived from young donors of eight mammalian species (365). In this study, fibroblasts grown from the dermis of hamster, rat, marmoset, rabbit, sheep, pig, cow and human, were assayed for resistance to cell death caused by 4 agents that induce oxidative stress and an agent that alters intracellular pH: H_2O_2 (which causes hydroxyl radical formation), paraquat (which induces intracellular superoxide formation), sodium arsenite (a heavy metal that induces intracellular free radical formation), *tert*-butyl hydroperoxide (which causes lipid peroxidation), and sodium hydroxide (a base). For each agent tested, there was a significant positive correlation between resistance to the agent (as measured by LD90, the dose required to kill 90% cells in the assay) and the maximum longevity of the donor species. Because the donor species were still quite evolutionarily diverse among the mammals, these data were also analyzed using a statistical adjustment to account for phylogenetic groupings of donor species and most of their results remained significant; the association between longevity and *tert*-butyl hydroperoxide was still positive, but no longer significant. However, this work too failed to address the issue of body size and metabolism on their observed regressions. The importance of this issue is illustrated by a report that among cells derived from mammalian species with differing lifespan, the replicative capacity of cell lines is positively correlated with donor size and not with donor longevity (366). Thus, complicating factors such as body size, metabolism, and phylogeny must be addressed in any interpretation of comparative biology studies.

In addition to the mechanisms of oxidative stress, there has been interest in the idea that organisms achieve long life by means of greater DNA repair capacity. Some of

the earliest work addressing the cellular mechanism of DNA repair across species was performed by Hart and Setlow (367). In this report, the ability of cells to repair UV-induced lesions as measured by unscheduled DNA synthesis (UDS) was tested in fibroblast lines from 7 different mammalian species with maximal lifespans from about 2 years (shrew) to greater than 100 years (human). They found that cells from long-lived species had both higher rates of UDS and a greater extent of UDS (i.e., more repair was performed over time) than did cells from short-lived species. Thus, these authors concluded that there was a positive correlation between rate of UV excision repair and longevity and suggested that long-life might then be regulated by genome integrity. Similar results on repair of UV lesions have been recapitulated in cells from differing numbers of mammalian species in fibroblasts (368-370), hepatocytes (371), lymphocytes (369), and lens epithelial cells (372). Of note, the work by Hall et al. (369) utilized both fibroblasts and lymphocytes derived from species all from a single order, primates, and found a strong positive correlation between rate of UDS and longevity. These results are of interest for two reasons: first they show that the long-lived animals have greater DNA repair capacity in multiple cell types, and second they show this relationship exists among closely related species even when the species compared are drawn from the same order of mammals.

The mechanisms responsible for repair of other forms of DNA damage also seem to be positively correlated with longevity. A study using mononuclear blood cells from 13 mammalian species found that maximum longevity was correlated with activity of poly(ADP-ribose) polymerase (PARP-1) (373). Following DNA damage by ionizing radiation, oxidation or alkylation, PARP-1 binds to single- and double-strand DNA breaks and catalyzes the posttranslational modification of proteins with a poly(ADP-ribose) moiety (374). The activity of PARP-1 is thought to regulate base excision repair, nucleotide excision repair, and non-homologous end-joining both by acting directly on proteins involved in repair and by relaxing chromatin near sites of DNA damage (374). Thus, increased activity of PARP-1 in longer-lived species would suggest that these animals have higher rates of repair of multiple forms of DNA damage. A second set of studies looked at the mutations formed by DMBA in skin- and lung-derived fibroblasts and found an inverse correlation between mutation rate and longevity of donor species

(375). Further, this report found that viability of cells following DMBA treatment was higher in cells from long-lived species, showing that stress resistance to DMBA was a function of DNA repair. There is some suggestion that this difference may be regulated by species-specific metabolic rates of DMBA conversion (376). Overall, these results suggest that evolution of long-lived species longevity may require co-evolution of multiple mechanisms that contribute to faithful maintenance of the genome.

Recently, there has been great interest in using the longest-lived rodent, the naked mole-rat (NMR), to address fundamental questions about the regulation of longevity. The NMR (*Heterocephalus glaber)* has been observed to live to ages of at least 28 years in captivity, about seven times longer than the maximum captive lifespan of laboratory mice (377). These animals are roughly the same size as mice, yet live about ten times longer than predicted by linear regression of lifespan against size for non-flying eutherians (357;378). NMR show little physiological or reproductive decline even when approaching 30 years of age, have never been observed to develop any spontaneous neoplasm, and do not show the typical age-associated acceleration in mortality risk that characterizes nearly every other species for which detailed survival data are available (377-381). Thus, physiological and biochemical mechanisms have evolved in this species that can dramatically extend lifespan compared to rodents of comparable size. Recent evidence also suggests that the extended lifespan of NMR is independent of several forms of oxidation resistance. Tissues from NMR exhibit activities of Sod1 and catalase that are similar to tissues from mice, and the activity of GpX is much lower than that of mice (382;383). Further, oxidative damage to proteins and nucleic acids and particularly to lipids in tissues from NMR are higher than those found in the same tissue from mice of the same chronological or physiological age (383;384). However, some tissues from the NMR seem to be resistant to oxidative injury, including arterial endothelial tissue and mitochondrial membranes from the liver and muscle (380;385). This potential paradox of long lifespan without particularly strong resistance to oxidative stress makes the NMR an interesting model in which to test hypotheses about the mechanisms of aging.

Clearly, much remains to be learned about mechanisms of stress resistance across species of differing lifespan. While the work of Kapahi and others has shown that cells

from long-lived animals are resistant, in culture, to oxidative stressors, there are essentially no data available on how similar cells respond to agents that cause injury by oxidation-independent mechanisms. And although there are a number of reports on the correlation between DNA repair rate and longevity, only one has attempted to show a functional association with stress resistance (375). These earlier reports have shown the applicability of cell culture models in exploring mechanisms of longevity across species. Thus, in this thesis we wished to test the hypothesis that species that have evolved long lifespan also exhibit cellular resistance to multiple forms of stress. To address many of the pitfalls discussed earlier, we based our study on a closely related group of mammals, i.e. the rodents, with differing lifespan, and included one long-lived member of another mammalian class (the little brown bat) for the sake of comparison. We also had an interest in comparing cellular stress resistance of the long-lived NMR with that of the mouse. The development of a model cellular system, like that proposed for Snell dwarf mice, could then facilitate further delineation of the mechanisms that regulate longevity across mammals.

Goals of the research thesis

The data presented in this introduction show that genetic perturbations in *C. elegans* and *Drosophila* that extend longevity also tend to render these animals resistant to lethal effects of multiple forms of stress. Thus, delineation of the mechanisms that regulate stress resistance in long-lived animals may provide insight into the molecular regulation of the aging process. Analysis of stress resistance in long-lived mice has given some hints that they, too, are resistant to stress, but the interpretation of these results is complicated due to the complexity of mammalian physiology and anatomy. The few cellular models that have been reported have suggested that regulation of longevity can affect resistance to stress at a cellular level. However, these reports have been limited in scope; few models have been utilized, resistance to few stressful agents have been tested, and further exploration into the biochemical and cellular processes regulating stress resistance has been virtually ignored (185;187;198;215;245).

The first goal of this thesis was to determine if fibroblasts derived from long-lived Snell dwarf mice were resistant in culture to the lethal effects of stress. The use of cell culture models eliminates the complexities of endocrine and neurological control in the whole animal, to thus focus attention on questions of cell biology. In addition, evaluation of cellular stress resistance provides a means to compare resistance of cells from a single animal to multiple types of stress in parallel. If, as some have predicted, stress resistance is a determinant of longevity, other long-lived mice should also exhibit cellular resistance to stress. Therefore, an extension of the first thesis goal was to determine if other cells from long-lived models, including other genetic mutants, are resistant to stress.

As discussed above, some evidence suggests that accumulation of DNA damage may regulate the rate of aging. This idea suggests that long-lived animals may have relatively stronger mechanisms for repairing DNA damage, which may in turn increase resistance to genotoxic stress. Thus, a second goal of this thesis was to determine if cells from long-lived Snell dwarf mice differ in their DNA repair capabilities relative to cells from control mice.

A third goal of the thesis was to address the idea that stress resistance may contribute to the evolution of long-lived species. It is an open question whether the aging process is regulated in a similar manner across animal species or if mechanisms of aging are unique among closely related groups. Most research on aging and stress resistance has focused on the changes that accompany life extension by calorie restriction or genetic mutation within a single species, and it is mostly unknown if the differences in maximum lifespan among different species of mammals is regulated by the same processes. It might be that throughout evolution, long lifespan has always generated cellular resistance to stress. A final goal of this thesis was to test this idea by determining if cells from long-lived mammals are resistant to multiple forms of stress relative to cells from short-lived animals.

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CHAPTER II

FIBROBLAST CELL LINES FROM YOUNG ADULT MICE OF LONG-LIVED MUTANT STRAINS ARE RESISTANT TO MULTIPLE FORMS OF STRESS

Note: The Introductory Findings are a summary of material published in Murakami S., Salmon A. and Miller R.A. Multiplex stress resistance in cells from long-lived dwarf mice. FASEB J. 2003 Aug;17(11):1565-1566. The remainder of the chapter is material that was published in Salmon A.B., Murakami. S., Bartke A., Kopchick J.J., Yasumura K, and Miller R.A. Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. Am J Physiol Endocrinol Metab. 2005 Jul;289(1):E23-29.

Introductory Findings

Much of the work in this thesis has derived from initial findings from our laboratory that were generated by a former research associate in our laboratory, Shin Murakami, and I. The rationale for exploring resistance to stress in long-lived animals was discussed in Chapter I. The issues of interpretation of stress resistance results using whole mice were also discussed in Chapter I. Because of the complexities of measuring the effects of stressors in living mice, Dr. Murakami and I developed an assay in which cellular resistance to stress could be assayed in fibroblasts grown from the skin of young adult Snell dwarf mice and littermate controls. Skin-derived fibroblasts were chosen for a number of reasons, including the relative ease of culturing this cell type and the large numbers of cells that can be obtained through serial subculture for biochemical and cellular assays. In our initial report, we found that fibroblasts grown from Snell dwarf mice are resistant to cell death induced by agents including oxidative stressors, such as

hydrogen peroxide and paraquat, as well as stressors that may act, in part, independently of oxidative stress such as the heavy metal cadmium, DNA damage caused by UV light, and heat. A compilation of these results is presented in Table 2.1. The results for hydrogen peroxide, cadmium and UV light were replicated using two separate means of measuring cell survival; cells from Snell dwarf mice were more resistant to these agents as assessed either using an assay based on reduction of WST-1, a tetrazolium salt that is reduced by living cells, or assessed by uptake of ^{3}H-thymidine uptake into replicating DNA. Further, we reported that resistance of Snell dwarf fibroblasts was found in cells from mice of two different genetic backgrounds, showing that stress resistance is independent of mouse strain and suggesting that the Pit1 mutation, and the resultant hormonal alterations in the mouse, induce cellular resistance to stress. We concluded that stress resistance of Snell dwarf fibroblasts represents an epigenetic change induced on the cells in vivo, because these changes persist through multiple cell divisions in culture and because the activity of the gene product of *Pit1* is unlikely to have a specific regulatory role in skin-derived fibroblasts.

Thus, the changes during the early development of Snell dwarf mice that lead to long-life also confer resistance to at least one cell type, resistance that can be measured even after multiple rounds of proliferation in long-term cell culture. Fibroblasts may then provide a convenient resource in which to develop hypotheses on the differential regulation of stress resistance in long-lived mutant mice. These results also support the hypothesis that Snell dwarf mice may contain other cell types that are resistant to stress in vivo, and that this resistance may contribute to the relative delay of aging phenotypes and extended longevity found in hypopituitary dwarf mice. Thus, defining the molecular basis for this coordinate control may direct research towards important insights into the regulation of aging in mice and other mammals, and by extension, provide biochemical targets for potential interventions. Therefore, the bulk of this thesis has been to utilize this cellular model to delineate the potential mechanisms that may regulate resistance to stress and in an effort to gain a better understanding of the underlying mechanisms of the aging process.

Abstract

Previous studies have shown that dermal fibroblast cell lines derived from young adult mice of the long-lived Snell dwarf mutant stock are resistant, in vitro, to the cytotoxic effects of hydrogen peroxide, cadmium, ultraviolet light, paraquat, and heat. We show here that similar resistance profiles are seen in fibroblast cells derived from a related mutant, the Ames dwarf mouse, and that cells from growth hormone receptor null mice are resistant to hydrogen peroxide, paraquat, and UV but not to cadmium. Resistance to UV, cadmium, and hydrogen peroxide are similar in cells derived from one-week old Snell dwarf or normal mice, and thus the resistance of cell lines derived from young adult donors reflects developmental processes, presumably hormone-dependent, that take place in the first few months of life. The resistance of cells from Snell dwarf mice to these stresses does not reflect merely anti-oxidant defenses: dwarf-derived cells are also resistant to the DNA-alklyating agent methyl methanesulfonate. Furthermore, inhibitor studies show that fibroblast resistance to UV light is unaffected by anti-oxidants ascorbic acid and N-acetyl-L-cysteine. These data suggest that postnatal exposure to altered levels of pituitary hormones leads to development of cellular resistance to oxidative and non-oxidative stressors which are stable through many rounds of in vitro cell division and could contribute to the remarkable disease resistance of long-lived mutant mice.

Introduction

The ability of an organism to respond to environmental and cytotoxic stresses may play an important role in regulation of the aging processes. Recent studies on the genetics of aging in the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster* have shown a positive correlation between resistance to stress and extended longevity. Genetic manipulations and environmental selections that increase life span in these models tend to enhance resistance to such stressors as reactive oxygen species, UV light, heavy metals and heat,

suggesting that the ability to respond to stress may be an important determinant of life span (1-8).

There is now a growing pool of evidence to suggest that stress resistance, at the cellular level, may also contribute to longevity in mammals (9-14). Many of these studies show that the animals are resistant to stressors in vivo, measured either through animal death or through modulation of stress response elements (12-16). However, homeostatic mechanisms within whole animals make results from such studies difficult to interpret. Recent studies have suggested that cells isolated from long-lived animals may retain properties of cellular resistance to stress in culture (12;13;17). Such results suggest that studies of cellular resistance to stress may help to delineate the mechanisms that may regulate both stress resistance and the aging process.

Our own prior investigations involved analysis of fibroblast cell lines derived from biopsies of the dermis of young adult mice of the Snell dwarf stock (17). Snell dwarf mice (dw/dw) have a mutation in the gene encoding a transcription factor, Pit-1, that results in abnormal pituitary development and an altered hormone profile featuring primary deficiencies in growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin, and thus secondary deficiencies in insulin-like growth factor I (IGF-I) and thyroxine. Snell dwarf mice live 40% longer than littermate controls and show delays or deceleration of multiple aspects of aging, including arthritis (18), collagen cross linking and T cell subset changes (19), and cancer (20;21), as well as delays in development of cataracts and glomerular pathology (21). Fibroblast cell lines derived from young adult Snell dwarf mice were found to be resistant to death induced by exposure to cadmium, hydrogen peroxide, paraquat, UV light, and heat compared to fibroblasts from control mice of normal life span (17). These results thus suggested a parallelism with previous work in *C. elegans*, in which perturbations to the IGF/insulin signaling pathway result in extended longevity and organismal resistance to stress (6;7;22-25).

The previous experiments on fibroblasts from Snell dwarf mice suggest the hypothesis that cellular stress resistance may contribute to the disease resistance and longevity seen in vivo in mutant mice with alterations in the IGF-I pathway, such as the Ames dwarf mice (Prop-$1^{df/df}$), and in mice deficient in the growth hormone receptor (GHR-KO mice) (26-28). To test this idea, we have now evaluated stress resistance in

cell lines derived from young adult mice of these long-lived models. In addition, we have conducted experiments to determine whether stress resistance is present in cells from neonatal mice or is acquired in the context of postnatal development, and have tested whether stress resistance of Snell dwarf fibroblast lines is or is not due simply to resistance to oxidative damage.

Methods

Animal subjects

Snell dwarf animals (and heterozygote controls) were dw/dw mice bred as the progeny of (DW/J × C3H/HeJ)-dw/+ females and (DW/J × C3H/HeJ)F1-dw/dw males. These sires had been previously treated with growth hormone and thyroxine to increase body size and fertility. Littermates with the (+/dw) genotype were used as controls. Tail skin biopsies were taken from male mice 4-5 days of age, or from male mice 3-4 months of age.

Ames dwarf mice and growth hormone receptor null or knockout (KO) mice (and littermate controls) were kindly provided by Dr. Andrzej Bartke at Southern Illinois University (Springfield, IL), from breeding stock originally generated by Dr. Kopchick's group at Ohio University. Tail skin biopsies from these mice were obtained from 3-6 month old males and sent overnight on ice in DMEM supplemented with 20% heat-inactivated fetal calf serum, antibiotics, fungizone and 10 mM HEPES. Fibroblasts were prepared from the biopsy tissue as described below. Ames dwarf (df/df) and control (+/+ or +/df) mice were produced by crosses between df/+ parents or between fertile df/df males and df/+ females. These mice were produced in a random-bred, closed colony with a heterogeneous background, which has been maintained for more than 20 years. GHR-KO mice and normal littermate controls were produced by mating heterozygous (+/-) carriers of the disrupted GHR/GHBP gene or homozygous knockout (-/-) males with (+/-) females. The genetic background of these animals is derived from 129/Ola embryonic stem cells and from BALB/c, C57BL/6, and C3H inbred strains.

(C57BL/6J x BALB/cJ)F1 hybrid (CB6F1) animals were obtained from the Jackson Laboratory (Bar Harbor, ME). Tail skin biopsies were taken from 3-4 month old males.

General procedure

Tail skin biopsies approximately 3-5 mm in length were obtained from the last ½ of the intact tail of isofluorane-anesthetized mice after skin sterilization with 70% ethanol. Biopsies were further washed in 70% ethanol, placed in Dulbecco's modified Eagle medium (DMEM, high-glucose variant, Gibco-Invitrogen, Carlsbad, CA), diced to less than 0.5 mm and digested overnight with collagenase type II (400 U/ml, 1000 U total per tail, Gibco-Invitrogen, Carlsbad, CA) dissolved in DMEM supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Sigma, St. Louis, MO) and 0.25 µg/ml of fungizone (Biowhittaker-Cambrex Life Sciences, Walkersville, MD) at 37º in a humidified incubator with 5% CO_2 in air. After collagenase treatment, cells were dislodged from digested tissue by repeated pipetting and passed through sterile nylon netting into sterile 14 ml centrifuge tubes (BD Dickenson, Bedford, MA). Samples were centrifuged 5 minutes at 200 *g* and collagenase solution was drawn off the cell pellet. Cells were resuspended in DMEM with 20% heat-inactivated fetal bovine serum, antibiotics and fungizone. Approximately 2.5 x 10^5 cells in 3 ml media were seeded into tissue culture flasks of 25 cm^2 surface area (Corning Costar, Corning NY). After 3 days, approximately 2/3 total volume of media was removed and replaced with fresh DMEM with 20% heat-inactivated fetal bovine serum, antibiotics and fungizone. Six or seven days after seeding, initial cultures (designated as first passage cells) were either (1) split six-fold or nine-fold by volume to create second passage cells, with twofold or threefold dilutions at each subsequent passage or (2) split and seeded at a density of 1 x 10^5 cells/cm^2 flask surface area at each passage into tissue culture flasks of 75 or 175 cm^2 surface area (Corning Costar). Cells were split by first washing flasks with 1X Phosphate buffered saline solution (PBS, 8.8 g NaCl, 2.25 g Na_2HPO_4 and 0.26 g NaH_2PO_4 per 1 L distilled H_2O, pH 7.3), followed by incubation

with approximately 3 ml trypsin/100 cm^2 surface area of flask 1X Trypsin-EDTA (Gibco-Invitrogen) for approximately 5 minutes at 37º in a humidified incubator with 5% CO_2 in air. Trypsin activity was inhibited with equal volume of DMEM with 20% heat-inactivated fetal bovine serum, antibiotics and fungizone. Subsequent passages were split at 6 day intervals, with approximately 2/3 total volume of media removed at day 3 and replaced with fresh DMEM with 20% heat-inactivated fetal bovine serum with antibiotics and fungizone. At the end of the third passage (6 days after seeding), confluent cells were used for assessment of stress resistance.

Assessment of cytotoxicity after exposure to stress

Six days after seeding, third passage cells were trypsinized as described. Cells were counted by hemocytometer and diluted to a concentration of 3 x 10^5/ml in DMEM with 20% fetal bovine serum with antibiotics and fungizone and seeded into a 96-well tissue culture-treated microtiter plate at a volume of 100 µl per well. After an 18-hour overnight incubation, cells were washed with 1X PBS and incubated in DMEM supplemented with 2% bovine serum albumin (BSA, Sigma), antibiotics and fungizone for approximately 24 hours. Previous work has shown (17) that this period of incubation in serum-starved conditions is critical for showing differences between mutant and control cells, because the presence of serum greatly increases stress-resistance of cells from both kinds of mouse. Cells were then exposed to a range of doses of one of the cytotoxic stressors. For UV light testing, cells were washed, then irradiated with UV light (254 nm at 5.625 J/m^2/s) in 100 µl of Dulbecco's PBS (Biowhittaker-Cambrex Life Sciences). Cells were then incubated in DMEM supplemented with 2% BSA, antibiotics and fungizone, and their survival was measured 18 hours later by a test based on oxidative cleavage of the tetrazolium dye WST-1 (Roche Applied Science, Indianapolis, IN) to a formazan product using the protocol suggested by the manufacturer. For assessment of resistance to H_2O_2, paraquat (methyl viologen), or cadmium (Sigma), the cells in the 96-well plates were incubated with a range of doses of stress agent for 6 hours in DMEM. Cells were then washed and incubated with DMEM supplemented with 2%

BSA, antibiotics and fungizone, and survival was measured 18 hours later by the WST-1 test. For assessment of resistance to methyl methanesulfonate (MMS), and actinomycin D (Sigma), cells in 96-well plates were incubated with a range of doses of each agent in DMEM for 24 hours. Each agent was administered as a stock solution in dimethylsulfoxide (DMSO, Sigma), and an equivalent level of DMSO (0.5% final concentration) was added to control cultures. Cells were then washed and incubated with DMEM supplemented with 2% BSA, antibiotics and fungizone, and survival was measured 18 hours later by the WST-1 test. All incubations were at 37º in a humidified incubator with 5% CO_2 in air.

Calculation of LD_{50} and statistical analysis

At each dose of chemical stressor, mean survival was calculated for triplicate wells for each cell line. The LD_{50}, i.e. dose of stress agent that led to survival of 50% of the cells, was then calculated using probit analysis as implemented in NCSS software (NCSS, Kaysville, UT). For this analysis, extremely low doses of stress agents that caused no cell death in fibroblasts from normal animals, as measured by WST1 assay, were removed from all data sets. Differences between groups in mean LD_{50} levels were evaluated by paired t-test, by t-test or by ANOVA, depending on the experimental design, with each day's work containing equal numbers of cultures from mutant and control, or equal numbers of cultures tested with each treatment for anti-oxidant studies.

Anti-oxidant treatment

Tests for anti-oxidant effects were conducted using the method described above for stress exposure, except that ascorbic acid (Sigma) or N-acetyl-L-cysteine (NAC, Sigma) was added during the 24 hr period prior to exposure to hydrogen peroxide, cadmium, paraquat, or UV light. Ascorbic acid was administered as a stock solution in DMSO, and an equivalent level of DMSO (0.5% final concentration) was added to control cultures. Final media and anti-oxidant solutions were brought to a pH of 7.2 by

addition of NaOH (Sigma). Ascorbate or NAC remained present during the exposure to the stress agent, after which cells were washed, incubated for 18 hr in DMEM with BSA, and tested for survival using the WST-1 procedure.

Results

To determine whether the association between lifespan and cellular resistance to stress extends to mouse mutant models of extended longevity other than Snell dwarf mice, we used a stress assay to test fibroblasts from Ames dwarf mice and GHR-KO mice. The results (Figure 2.1) revealed that fibroblasts grown from homozygous Ames dwarf mice (df/df) are significantly more resistant to UV light, H_2O_2, and cadmium than fibroblasts derived from normal littermate controls. The effect sizes range from 43% (UV) to 95% (cadmium) and all results were significant by paired t-test at $P<0.05$. The data also suggest that fibroblasts from Ames dwarf mice may be slightly more resistant to paraquat, although not significantly, in a study with a sample size of 6 per group.

Fibroblasts from mice lacking growth hormone receptor (GHR-KO) were significantly more resistant to H_2O_2, UV light, and paraquat when compared to fibroblasts from littermate control mice (Figure 2.2). Effect sizes ranged from 47% (paraquat) to 194% (UV light) and were all significant by paired t-test at $P<0.05$. There was, however, no evidence for cadmium resistance in fibroblasts from GHR-KO mice.

Newborn mice of the Snell and Ames genotype and GHR-KO mice are indistinguishable in size from control littermates (26;29), but begin to show growth retardation in the first week of life as a consequence of their pituitary hormone deficiencies (Ames, Snell) or diminished response to GH (GHR-KO). To determine whether differential resistance to stress was present in neonates, or alternately developed gradually after birth, we tested cell lines derived from tail biopsies from newborn (4-5 days old) Snell dwarfs and controls in parallel with lines taken from separate mice that were young adults (3-4 months old). The results are shown in Table 2.2. Consistent with previous findings, cells derived from young adult Snell dwarf mice show significantly greater resistance to UV light, H_2O_2, and cadmium. In contrast, fibroblasts from neonatal Snell dwarfs are not more resistant than control lines to UV or cadmium. Fibroblasts

from neonatal dwarf mice are significantly more resistant to hydrogen peroxide than controls, but the effect size (32%) is substantially less than that seen for lines derived from young adult mice (61%). These data suggest that development of stress resistance depends upon developmental changes, presumably hormone dependent, that occur in the first few months of postnatal life.

The resistance of Snell dwarf cell lines to multiple forms of stress is consistent with either of two models. It may be that each of the test agents injures cells principally via oxidation damage, to which dwarf-derived cells are particularly resistant. Alternately, dwarf fibroblast lines may be resistant to multiple forms of injury, only some of which involve oxidation damage. To begin to discriminate between these ideas, we evaluated cell injury induced by hydrogen peroxide, cadmium, paraquat, and UV light in the presence of ascorbic acid (Vitamin C) or NAC (N-acetyl-L-cysteine). Results are shown in Table 2.3 for ascorbic acid and Table 2.4 for NAC. In each case the cells were incubated with the anti-oxidant both for 24 hours prior to stress exposure (during the period of serum deprivation) and then also during exposure to the stress. As expected, ascorbic acid protected against H_2O_2-mediated cell death ($F = 10.1$, $P < 0.001$), and also against paraquat ($F = 8.8$, $P < 0.001$), which is thought to act by production of intracellular free radicals (30). The greater degree of protection against hydrogen peroxide may reflect inactivation of hydrogen peroxide prior to its entry into the fibroblast cytoplasm. Ascorbic acid treatment also gave significant protection from cadmium-mediated cell death ($F = 5.8$, $P = 0.008$), though to a lower degree, consistent with previous reports that cadmium-mediated cytotoxicity involves, at least in part, oxidative damage (31). Ascorbic acid did not, however, provide any protection from cell death caused by UV light irradiation (Table 2.3, $F = 1.2$, $P = 0.321$). Table 2.4 shows similar results using an alternate anti-oxidant, NAC, that provided significant protection against cell injury produced by hydrogen peroxide ($F = 17.3$, $P < 0.001$) and cadmium ($F = 5.1$, $P = 0.015$), consistent with the ascorbate results. The high level of protection against cadmium may be an artifact related to the chelation of cadmium ions by NAC (32). NAC provided protection from paraquat induced cell death, though these data did not reach significance ($F = 0.8$, $P = 0.472$). Once again, though, cytotoxicity induced by UV light was not altered by the presence of an anti-oxidant ($F = 0.2$, $P = 0.832$),

suggesting that the protective effect seen in Snell dwarf cell lines was not restricted to agents that induce oxidative damage alone.

To determine whether fibroblasts from long-lived mice are resistant to other forms of cell death that do not involve direct oxidative damage, we tested the effect of methyl methanesulfonate (MMS) and actinomycin D on fibroblasts from Snell dwarf mice and normal littermate controls. MMS is a monofunctional DNA alkylating agent and a known carcinogen (33;34) and primarily methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine (35). Although the N^7-methylguanine adduct may be nontoxic and nonmutagenic, N^3-methyladenine is a lesion that inhibits DNA synthesis and needs to be actively repaired to prevent cell death (34;36). Actinomycin D is an antineoplastic antibiotic that inhibits cell proliferation by forming stable complexes with DNA and blocking the movement of RNA polymerase, interfering with RNA synthesis and inducing apoptosis (37). Both agents induced dose-dependent cell death in dermal fibroblast cell lines from both genotypes as measured by WST1. In cultures isolated from 12 Snell dwarf animals and 11 normal littermates, dwarf fibroblasts were significantly more resistant to cell death induced by MMS (Figure 2.3). There was, however, no difference in resistance of these cultures to cell death induced by actinomycin D (Figure 2.3).

Discussion

Our previous work has shown that fibroblasts isolated from long-lived Snell dwarf mice are more resistant to multiple cytotoxic stressors than are fibroblasts from control mice of normal life span (17). The results presented here replicate the earlier data, and show also that stress resistance is seen in fibroblasts from phenotypically similar mouse mutants with extended longevity, i.e in Ames dwarf and GHR-KO mice. We also show that this stress resistance is not present at birth, but is acquired in the first few months of life, and that resistance is seen not just to oxidative insults, but also to agents like UV light and MMS which interfere with cellular functions principally through other means.

Our results extend the evidence for the hypothesis that mutations that extend lifespan may do so by increasing the resistance of cells to stress. The Ames dwarf and the Snell dwarf have different mutations within a shared pathway critical to development of the embryonic pituitary gland. As a result, mice with these mutations share an altered hormonal profile that features declines in GH, TSH, prolactin, IGF-I and thyroxine. The similarities in stress resistance profiles of fibroblasts from Snell and Ames dwarf mice not only confirm our earlier findings in the Snell mutant, but also show that the association of cellular resistance to organismal longevity is replicable on different genetic backgrounds and on mice raised in different vivaria. GHR-KO mice differ in their hormonal profile from Ames and Snell dwarf mice; the GHR-KO mice have high levels of GH, modest declines in thyroid hormones, and very low IGF-I levels (26-28). Thus the observation that resistance to hydrogen peroxide, paraquat, and UV light is seen in cells from the GHR-KO mice indicates that IGF-I deficits may underlie fibroblast cell resistance to these three agents. The absence of evidence for cadmium resistance in GHR-KO cell lines suggests that resistance to this agent may depend on hormonal, or perhaps other, factors that differ between the pituitary mutants and the GHR-KO model.

Damage caused by reactive oxygen species, accruing over a lifetime, has been postulated to be an important determinant of lifespan both in normal individuals (38;39) and in animal models that show extended longevity (40-42). This hypothesis is supported by evidence that levels of anti-oxidants are higher in tissues of Ames dwarf mice (43;44), although results from GHR-KO mice are less clear (45). To a limited extent, our results are consistent with this idea, by showing that fibroblasts from Ames dwarf, Snell dwarf and GHR-KO mice are relatively resistant to damage induced by H_2O_2 or paraquat. But the results with ascorbate and NAC inhibition, as well as the MMS data, clearly indicate that resistance of the fibroblasts cannot be attributed solely to differential vulnerability to reactive oxygen species. In our assay, the anti-oxidants ascorbic acid or NAC provide no protection from UV light, to which cells from all three mutant mice are resistant. In addition, Snell dwarf derived cell lines are resistant to MMS, whose injury reflects DNA alkylation (33;34). These data suggest that fibroblasts from dwarf mice are resistant to multiple forms of internal damage, only some of which are mediated by reactive oxygen species. One potential complication of our design is that our standard culture conditions

expose the cells to atmospheric oxygen levels, approximately 20% in air, which are higher than typical tissue oxygen levels (46) and could potentially induce either oxidative damage, or compensatory responses to damage, that would not have been seen in cells grown under lower oxygen tension. Atmospheric levels of oxygen have previously been shown to induce cellular damage, particularly to DNA, that can induce cell senescene in culture (47;48). Scott Maynard, a former postdoctoral researcher in our lab, has shown that there is no difference between Snell dwarf- and control-derived fibroblasts in their resistance to paraquat or H_2O_2 when cells are grown in culture conditions using 3% oxygen in air, rather than the standard 20% oxygen in air (49). Further, Maynard showed that fibroblasts from control mice undergo cell senescence earlier than those from Snell dwarf mice when grown in 20% oxygen culture conditions, suggesting that Snell dwarf cells might accumulate fewer, or repair more effectively, oxidative DNA lesions that lead to cell senescence (49).

The involvement of IGF-I in our stress resistance system is likely to be indirect. Although IGF-I levels in culture do modulate cell proliferation and stress resistance (50-53), our stress assays are conducted on cell lines that have been grown in defined culture medium, containing serum, for at least three passages (about 3 weeks in vitro) prior to the withdrawal of serum and exposure to stress. Thus it seems likely that the stress resistance phenotypes are regulated by developmental differences in exposure to hormones, presumably including IGF-I, during the development of the mouse prior to tissue biopsy (54). The epigenetic changes induced in vivo might then be retained in cells isolated from these mutant mice.

Our data showing that cell lines derived from mice 4-5 days of age show no genotypic effect on resistance to UV or cadmium, and only a small difference in hydrogen peroxide resistance, are consistent with this interpretation. The pre-natal environment for both Snell dwarf and littermate control mice is identical, as both develop in the same uterus with hormones and nutrients provided through the maternal circulation. Snell dwarf and normal littermates are indistinguishable in size at birth, but thereafter Snell dwarf mice grow more slowly than normal mice (26). Similarly in *Drosophila*, alterations in early life growth due to differences in hormonal signaling,

either through genetic mutation or experimental manipulations, tend to be correlated with extended life span and increased resistance to stress (55-58).

Our data suggest that cells from long-lived mutant mice may be more resistant to direct DNA damage, whether induced directly by UV light and MMS, or perhaps indirectly by exposure to H_2O_2, paraquat and cadmium. Such resistance might also contribute to the relative delay in cancer incidence rates in these animals (20;21). It will be interesting to examine levels of specific forms of DNA damage and repair systems in fibroblasts from long-lived mice and in cells within the intact animal. More work is also needed to define the relationship between cellular stress resistance and proteins such as p53 and mammalian Sir2 homologues that participate in DNA damage response pathways, and to proteins that control stress induced changes in cell cycle regulation, such as FOXO (59-63).

We should also mention the possibility that fibroblasts isolated from dermis may represent a heterogeneous collection of cells with overlapping properties (64), and that these populations may differ between cells from dw/dw and dw/+ mice. It will take additional studies to determine whether the differences seen between fibroblasts of dwarf and control mice represent changes in all fibroblasts isolated for each sample, or a result of changes in the relative proportion of cells with stress-resistant properties (65;66).

Dermal fibroblasts provide convenient in vitro models for cellular and molecular studies, but are unlikely to regulate aging and late life pathology in mammals. It will thus be useful to measure cellular stress resistance in vivo, using systems that evaluate liver function in response to an inducer of oxidative stress such as acetaminophen (67) or the resistance to kainic acid-induced lesions and excitotoxic neurodegeneration of cells within the hippocampus (68). Studies of fibroblasts from long-lived rodents may also provide new insights into the possible role of stress resistance in modulating longevity among closely related species (69;70).

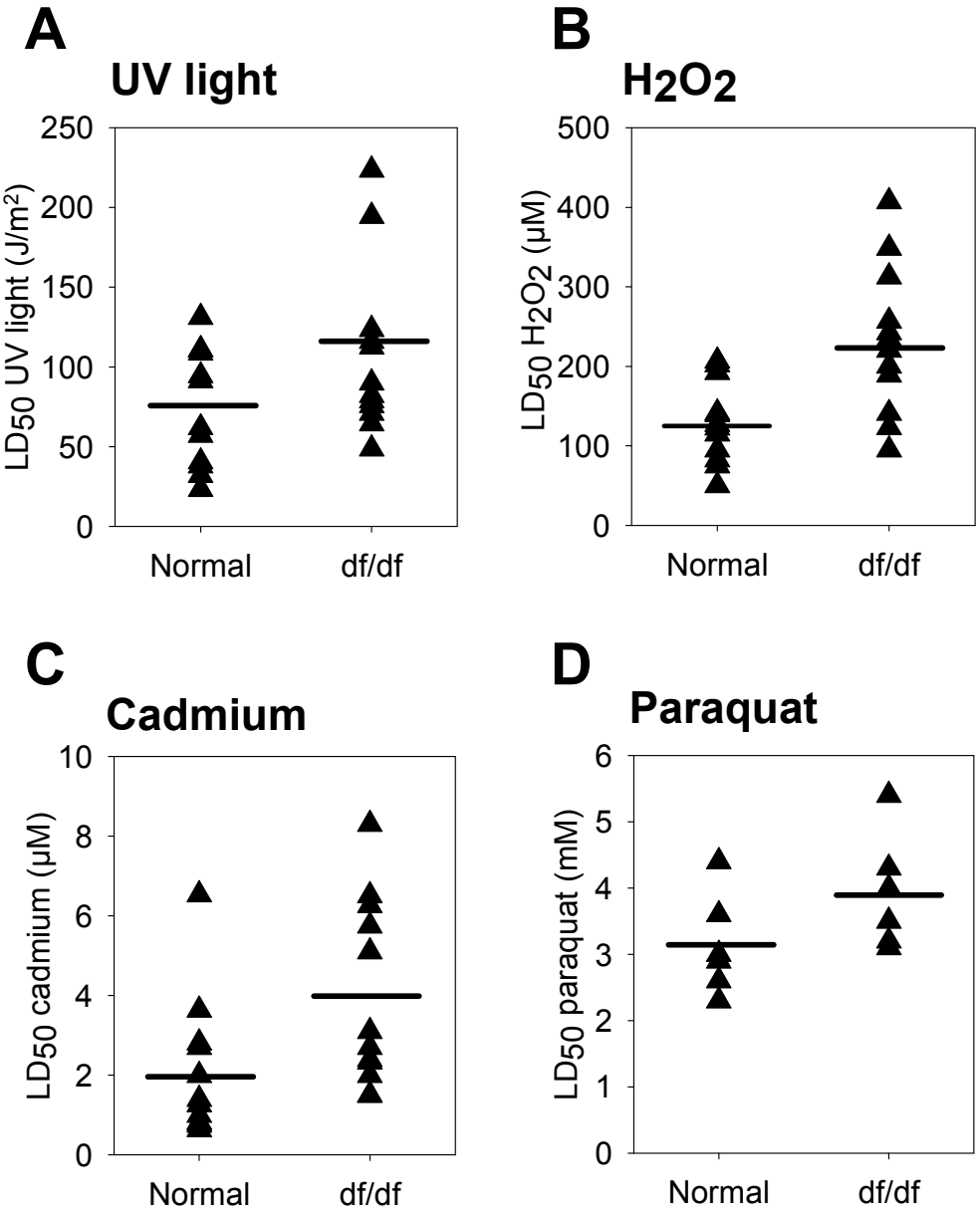

Figure 2.1. Skin-derived primary fibroblasts from Ames dwarf mice are resistant to multiple forms of stress compared to control. Each symbol in the figure represents fibroblasts isolated from an individual mouse of the indicated genotype; the horizontal line indicates the mean value for each group. Cell survival was measured by conversion of WST-1. Percent increases in LD_{50} and p-values for paired t-test were as follows: A. UV light (43%; *P*=0.017, n=12); B. hydrogen peroxide (79%; *P*=0.002, n=12); C. cadmium (95%; *P*=0.021, n=12); D. paraquat (25%, *P*=0.141, n=6).

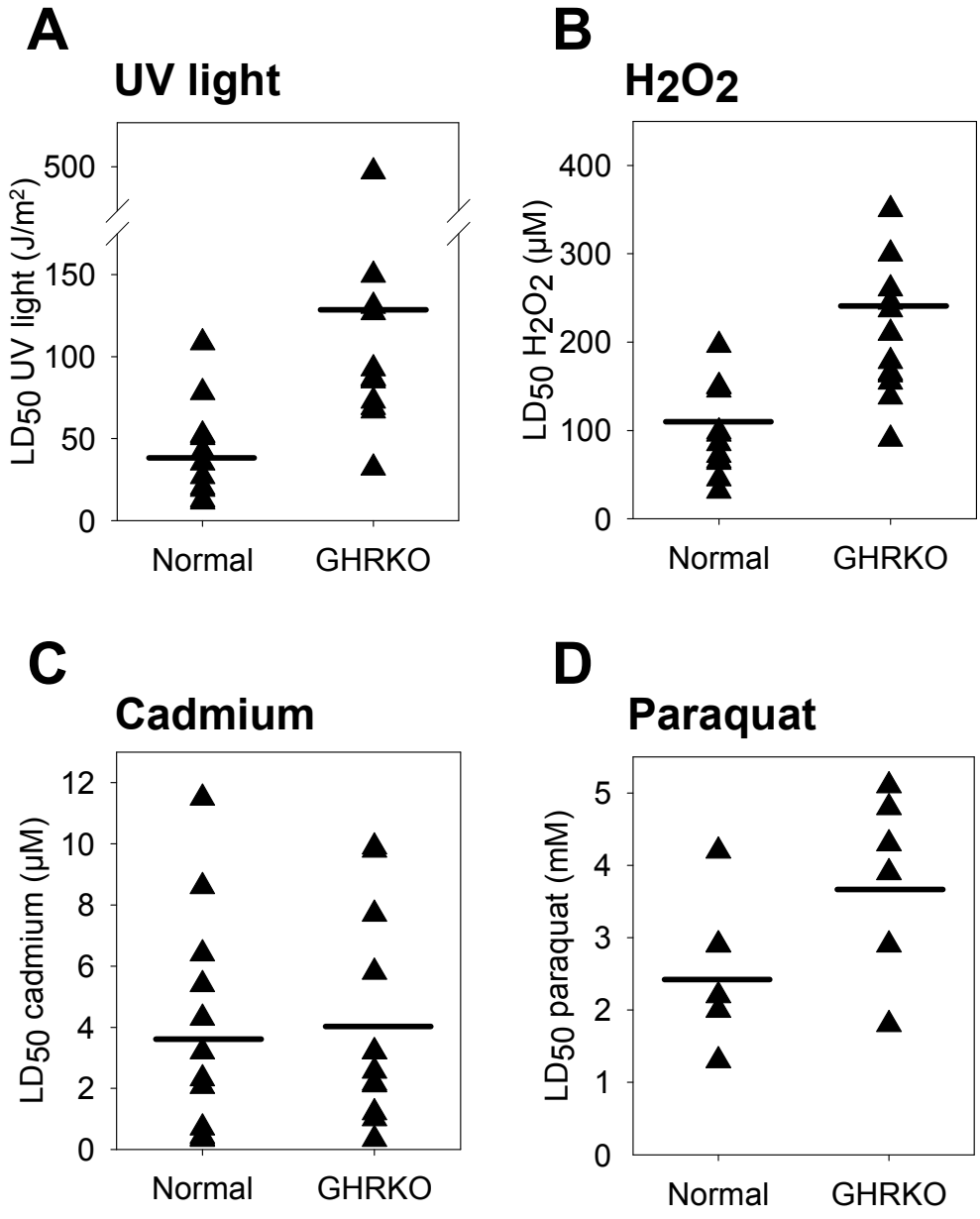

Figure 2.2. Skin-derived primary fibroblasts from growth hormone receptor knockout (GHR-KO) mice are resistant to UV, H_2O_2 and paraquat, but not to cadmium. Each symbol in the figure represents fibroblasts isolated from an individual mouse of the indicated genotype; the horizontal line indicates the mean value for each group. Cell survival was measured by conversion of WST-1. Percent increases in LD_{50} and p-values for paired t-test were as follows: A. UV light (194%; *P*=0.049; n=12); B. hydrogen peroxide (108%; *P*=0.002; n=12); C. cadmium (7%; *P*=0.36; n=12); paraquat (47%; *P*=0.016; n=6).

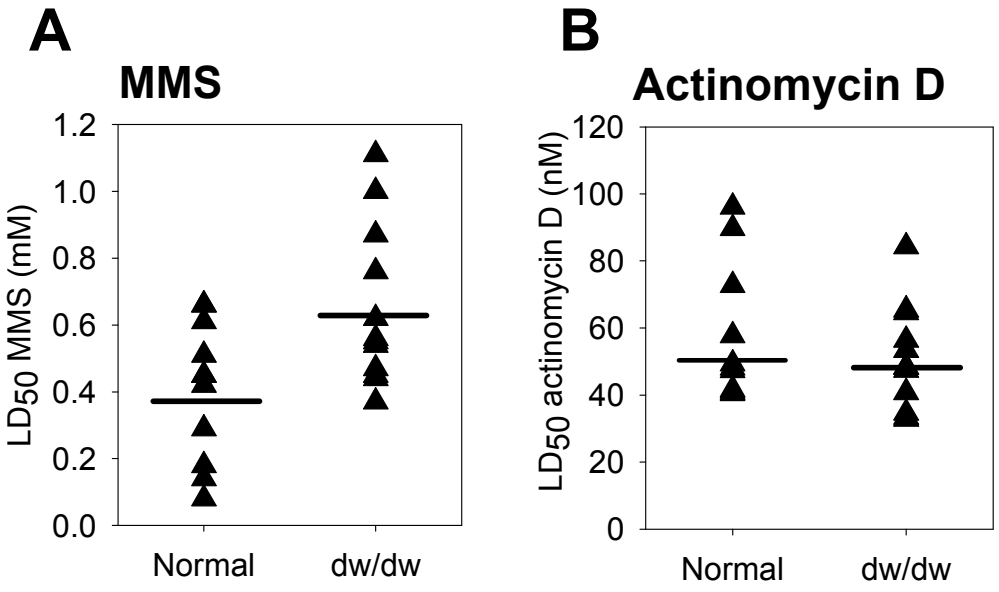

Figure 2.3. Skin-derived primary fibroblasts from Snell dwarf mice are resistant to cell death induced by methyl methanesulfonate, but not to cell death induced by actinomycin D. Each symbol in the figure represents fibroblasts isolated from an individual mouse of the indicated genotype; the horizontal line indicates the mean value for each group. Cell survival was measured by conversion of WST-1. Percent increases in LD_{50} and p-values for t-test were as follows: A. methyl methanesulfonate (60%; P=0.017, n=12 dw/dw, 11 dw/+); B. actinomycin D (-10%; P=0.43, n=12 dw/dw, 11 dw/+).

Table 2.1. Stress resistance of fibroblasts from normal and Snell dwarf mice.

Stress	Strain	LD_{50}	No. of mice	ANOVA *p* value	Increase
		Mean ± SD			
UV light	Normal	110 ± 19 (J/m^2)	10	-	-
	Dwarf	160 ± 29 (J/m^2)	13	< .0001	45%
H_2O_2	Normal	92 ± 23 (μM)	6	-	-
	Dwarf	227 ± 20 (μM)	8	.002	147%
Paraquat	Normal	1.28 ± 1.1 (mM)	7	-	-
	Dwarf	2.04 ± 0.9 (mM)	10	.01	53%
Cadmium	Normal	4.5 ± 3.8 (μM)	12	-	-
	Dwarf	12.6 ± 10.8 (μM)	15	.007	180%
Heat	Normal	14.1 ± 9.4 (min)	8	-	-
	Dwarf	28.6 ± 8.6 (min)	11	.006	102%

The table shows the Average LD_{50} across the set of mice tested. The LD_{50} was calculated for each mouse by probit analysis as implemented in NCSS statistical software. Probabilities were calculated by two-way analysis of variance (ANOVA), using genotype and assay date as predictor variables; the p-value shown represents the null hypothesis that genotype has no effect on LD_{50}. Cell survival was measured by conversion of WST-1.

Table 2.2. Stress resistance of fibroblasts from normal and Snell dwarf mice of different ages

	Stress	Strain	LD_{50}* Mean ± SE	Lines tested	T-test p-value	Differ.
Newborn						
	UV	Normal	109 ± 18 J/m^2	10	-	-
		Snell dwarf	83 ± 26 J/m^2	8	0.531	-24%
	H_2O_2	Normal	500 ± 76 μM	8	-	-
		Snell dwarf	666 ± 67 μM	6	0.007†	+32%
	Cadmium	Normal	10.9 ± 2.8 μM	9	-	-
		Snell dwarf	9.9 ± 2.4 μM	6	0.739	-9%
Young adult						
	UV	Normal	102 ± 18 J/m^2	7	-	-
		Snell dwarf	187 ± 39 J/m^2	7	0.021†	+83%
	H_2O_2	Normal	212 ± 46 μM	6	-	-
		Snell dwarf	341 ± 76 μM	6	0.032†	+61%
	Cadmium	Normal	5.7 ± 2.7 μM	7	-	-
		Snell dwarf	10.8 ± 3.7 μM	7	0.016†	+90%

Newborn refers to cells derived from skin biopsies collected from mice 4-5 days after birth. Young adult refers to cells derived from skin biopsies collected from mice 3-4 months old. Cell survival was measured by conversion of WST-1.
*Average LD_{50} across the set of mice tested.
†Difference between normal and dwarf significant by t-test.

Table 2.3. Stress resistance of fibroblasts exposed to ascorbic acid (Vitamin C)

Stress	Treatment	LD_{50}* Mean ± SE	Cultures tested	p-value†	Protection ‡
UV	Control	95 ± 13 J/m^2	10	-	-
	100 μM	68 ± 13 J/m^2	10	0.390	– 27%§
	1000 μM	105 ± 18 J/m^2	10	0.991	12%
H_2O_2	Control	136 ± 12 μM	15	-	-
	100 μM	340 ± 45 μM	15	0.082	150%
	1000 μM	551 ± 108 μM	15	<0.001	305%
Paraquat	Control	2.0 ± 0.2 mM	16	-	-
	100 μM	3.3 ± 0.2 mM	16	0.009	60%
	1000 μM	3.6 ± 0.4 mM	16	<0.001	78%
Cadmium	Control	2.4 ± 0.3 μM	10	-	-
	100 μM	3.2 ± 0.4 μM	10	0.224	32%
	1000 Mm	4.2 ± 0.4 μM	7	0.006	71%

*Average LD_{50} across the set of mice tested.
† For each stress agent an ANOVA calculation (one factor with three dose levels) was calculated, followed by the Tukey-Kramer post-hoc test. Tukey-Kramer p-values are shown for the null hypothesis that LD_{50}s for Vitamin C-exposed cells were equal to those in control cells. Cell survival was measured by conversion of WST-1.
‡Increase in resistance to stress as compared to control.
§Decrease in resistance.

Table 2.4. Stress resistance of fibroblasts exposed to N-acetyl-L-cysteine (NAC)

Stress	Treatment	LD_{50}* Mean ± SE	Cultures tested	p-value†	Protection ‡
UV	Control	99 ± 22 J/m^2	8	-	-
	1 mM	85 ± 17 J/m^2	8	0.846	– 14%§
	10 mM	87 ± 14 J/m^2	8	0.873	– 13%§
H_2O_2	Control	123 ± 24 μM	8	-	-
	1 mM	413 ± 38 μM	8	0.579	168%
	10 mM	1713 ± 350 μM	8	<0.001	1297%
Paraquat	Control	3.4 ± 1.0 mM	8	-	-
	1 mM	5.3 ± 1.9 mM	8	0.789	57%
	10 mM	7.0 ± 2.9 mM	8	0.439	107%
Cadmium	Control	1.7 ± 0.2 (μM)	8	-	-
	1 mM	7.4 ± 1.8 (μM)	8	0.637	340%
	10 mM	21.0 ± 7.4 (μM)	8	0.015	1154%

*Average LD_{50} across the set of mice tested.
† For each stress agent an ANOVA calculation (one factor with three dose levels) was calculated, followed by the Tukey-Kramer post-hoc test. Tukey-Kramer p-values are shown for the null hypothesis that LD_{50}s for NAC-exposed cells were equal to those in control cells. Cell survival was measured by conversion of WST-1.
‡Increase in resistance to stress as compared to control.
§Decrease in resistance.

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CHAPTER III

CORRELATED RESISTANCE BETWEEN CYTOTOXIC AGENTS AND GLUCOSE DEPRIVATION IN FIBROBLASTS FROM LONG-LIVED MICE

Note: This material is an excerpt of material published in Leiser, S.F., Salmon A.B., and Miller R.A. Correlated resistance to glucose deprivation and cytotoxic agents in fibroblast cell lines from long-lived pituitary dwarf mice. Mech Ageing Dev. 2006 Nov;127(11):821-829.

Abstract

Previous studies have shown that fibroblast cell lines derived from the skin of young adult mice of the long-lived Snell dwarf mutant mouse stock are resistant to the cytotoxic effects of multiple agents, including hydrogen peroxide, cadmium, heat, ultraviolet light, and the carcinogen methyl methanesulfonate. In this report, analysis of cell lines derived from three different strains of mice shows that cell lines resistant to peroxide-induced cytotoxicity were also relatively resistant to death induced by paraquat, cadmium, and ultraviolet light. In addition, we report that Snell dwarf fibroblasts are relatively resistant to the non-lethal effects induced by low glucose concentrations, and also to the effects of the mitochondrial poison rotenone, a blocker of Complex I of the electron transport chain. Resistance to the metabolic effects of low glucose medium was associated with resistance to peroxide and cadmium in cells from heterogeneous mice and Snell dwarf mice, though unexpectedly not associated with resistance to the lethal effects of paraquat. Further analysis of the basis for metabolic abnormalities in these cell lines may provide insights into the cause of stress resistance in dwarf-derived cultures and to the longevity and disease-resistance of these long-lived mutant mice.

Introduction

Mutation of the *Pit1* gene, when homozygous, leads to abnormalities in embryonic development of the anterior pituitary and thus to deficits in production of growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin in juvenile and adult mice. Deficits in GH and TSH lead to deficits in circulating levels of insulin-like growth factor I (IGF-I) and thyroxine, which in turn result in dwarfism, and thus young adult Snell dwarf mice are about one third the size of littermate controls (1-3). Snell dwarf mice have a greatly extended lifespan, with mean and maximal survival approximately 40% longer than littermate controls on a variety of backgrounds (3;4). The lifespan extension of the Snell dwarf mutant seems to represent a retardation of multiple forms of late-life pathology, including joint disease (5), immune and connective tissue changes (3), changes of the kidney glomerulus and eye lens and development of neoplasia (6). It seems likely that the anti-aging effects in Snell dwarf mice, and the phenotypically similar Ames dwarf mice, represent, at least partially, a consequence of diminished IGF-I levels, because life span extension is also seen in mice lacking the gene for the growth hormone receptor binding protein (GHR-KO), in which high GH levels accompany low IGF-I levels (7), as well as in mice with low IGF-I receptor levels (8) and in mice with absence of the growth hormone releasing hormone (lit/lit mice, mutant for Ghrhr) (3).

We have previously shown that early passage fibroblast cell lines derived from the skin of young adult Snell dwarf mice are resistant to multiple forms of lethal stress. These include agents that cause primarily oxidative damage, such as hydrogen peroxide and paraquat, as well as agents that kill cells through other pathways such as the toxic metal cadmium, heat, ultraviolet light, and the DNA alkylating agent methyl methanesulfonate (9;10). The differences in stress resistance between dwarf and control mice are weak or absent in cell lines derived from mice less than one week of age (10), and thus seem likely to reflect epigenetic changes induced when fibroblasts differentiate, in the skin, in a specific hormonal milieu. The unusually high stress resistance of cells from Snell dwarf mice persists in cultured cells for many weeks, at least through the fourth in vitro passage, and thus represents a stable characteristic of these cultures. We

have found that fibroblasts from the skin of young adult Ames dwarf mice and the GHR-KO mice are resistant to most of these cytotoxic stresses as well (10).

Thus, these data suggest that mutations that extend longevity in mice also confer cellular resistance to multiple stressors in parallel. Motivated by these findings, we have evaluated whether there is a correlation among resistance to different stressors. By assaying cell lines in parallel for their resistance to different agents, we have found a positive correlation between resistance to peroxide and resistance to each of paraquat, cadmium, and UV light. In a separate line of investigation, Scott Leiser, another graduate student in the lab, has found that fibroblasts from Snell dwarf mice are relatively resistant to the non-lethal effects of glucose withdrawal from the cell culture media and to incubation with rotenone, a mitochondrial poison that blocks an early step in the electron transport chain. We have found that these effects are negatively correlated with resistance to cadmium and to peroxide. Thus, these data suggest coordinate regulation of pathways that regulate not only resistance to cytotoxic agents, but also to resistance to metabolic inhibitors. Further, these data suggest that the hormonal alterations that extend lifespan in Snell dwarf mice also coordinately regulate these pathways such that cells from these long-lived mice are resistant to multiple forms of lethal and non-lethal stress.

Methods

Animal subjects

Snell dwarf (*dw/dw*) mice, and heterozygote (*dw/+*) controls were bred as the progeny of (DW/J × C3H/HeJ)-*dw/+* females and (DW/J × C3H/HeJ)F1-*dw/dw* males. These sires had been previously treated with growth hormone and thyroxine to increase body size and fertility. Tail skin biopsies were taken from male mice 3-6 months of age. (C57BL/6J x BALB/cJ)F1 hybrid (CB6F1) animals were obtained from the Jackson Laboratory (Bar Harbor, ME). Tail skin biopsies were taken from 3-6 month old males. UM-HET3 mice were produced as the offspring of (BALB/cJ x C57BL/6J)F1 (CB6F1) females and (C3H/HeJ x DBA/2J)F1 (C3D2F1) males; UM-HET3 tail skin biopsies were taken from 3-20 month old males.

Cell culture and stress assays

Isolation of primary fibroblasts from tail biopsy samples and subculturing was performed as previously described (9;10). Following subculture, second passage cells were assayed for resistance to lethal stressors as previously described (9;10). For glucose assays and rotenone assays, cells were plated as for lethal stress assays. For glucose assays, after an approximately 18-hour overnight incubation, cells were washed twice with 37° C 1X PBS and incubated in DMEM containing a range of glucose concentrations. These concentrations were made using media containing glucose-free DMEM, supplemented with 20% heat-inactivated dialyzed fetal bovine serum, antibiotics, fungizone, and supplemental glucose (Sigma, St. Louis MO). High-glucose DMEM (~4.0 mg/ml) with 20% heat-inactivated dialyzed fetal bovine serum (which contains very low levels of glucose), antibiotics and fungizone was used as a control. For rotenone assays, after an approximately 18-hour overnight incubation, cells were washed once with 37° C 1X PBS, and incubated in DMEM containing 20% fetal bovine serum, antibiotics, fungizone, and a range of doses of the mitochondrial inhibitor rotenone (Sigma) dissolved in DMSO, with DMSO only as a control. For all, cell viability or mitochondrial inhibition was measured by conversion of WST-1 formazan dye as previously described (9;10).

Calculation of LD50 and ED50 values, and statistical analysis

For calculation of the resistance of each cell line to chemical stressors, mean survival was calculated for triplicate wells at each dose of chemical stressor for each cell line. The LD50, i.e. dose of stress agent that led to survival of 50% of the cells, was then calculated using probit analysis as implemented in NCSS software (NCSS, Kaysville, UT). For this analysis, extremely low doses of stress agents that caused no cell death in fibroblasts from normal animals, as measured by WST1 assay, were removed from all data sets. ED50 values for glucose withdrawal and rotenone treatments were calculated in a similar manner. For assessment of correlations between cytotoxic stressors and glucose resistance, cultures for which at least 2 stressors were tested (or 1 stressor and

glucose resistance) were assayed separately by genetic strain (or mutant) with each day's work containing equal numbers of cultures from mutant and control for the dwarf studies. Because some data sets were found to be distributed non-normally, Spearman rank coefficients of correlation were used to assess to evaluate resistance to the different agents.

Results

Our previous work (9;10) has shown that fibroblast cell lines from Snell dwarf mice are resistant to multiple forms of lethal stress, including hydrogen peroxide, paraquat, cadmium, and UV light. The new results showing that these cell lines are also resistant to metabolic inhibition induced by low glucose concentration or by mitochondrial function inhibitors raised the suggestion that common pathways might modulate cell resistance to lethal stress as well as responses to low glucose and metabolic inhibition. To test this idea, we used a correlation approach to see if those cell lines with unusually high or low resistance to lethal stresses showed unusually high or low sensitivity to the effects of low glucose concentrations. Because we thought it plausible that both stress resistance and glucose sensitivity might be under genetic control, we conducted similar analyses using data from either isogenic mice (from the CB6F1 hybrid stock) or from a stock (UM-HET3) with controlled genetic heterogeneity. Mice from Snell dwarf and littermate control stocks, which show a limited degree of heterogeneity in their segregating background genes, were also evaluated in this way.

We began by tests of the hypothesis that cell lines resistant to one of the lethal stress agents would be relatively resistant to other stress agents in this class. The top line of Table 3.1 presents Spearman correlation coefficients from the UM-HET3-derived cell lines, which represent the largest degree of genetic heterogeneity. We find good evidence for correlation between resistance to H_2O_2 and each of the other agents tested, i.e. paraquat, cadmium, and UV. The correlation coefficient in each case is $R > 0.62$, and each is significant at a level of at least $p < 0.002$. It is in this context noteworthy that a previous study using anti-oxidants has shown that UV-induced cell death in our culture system is not modified by either Vitamin C or N-acetyl-cysteine, and thus presumably

reflects induction of cell death by a mechanism that does not involve oxidation damage (10). Scatterplots of these relationships are included in the top panels of Figure 3.1, to illustrate that the relationships do not reflect the undue influence of a small number of outliers.

Data from Snell dwarf mice and their non-mutant littermate controls are shown in the next two lines of Table 3.1. With one exception (responses of littermate controls to UV), each of these correlations is also positive with $R \geq 0.3$, and four of the correlations reach statistical significance. The responses of the Snell dwarf mice are also included in Figure 1 (middle row). Table 3.1 also includes summary statistics for the isogenic CB6F1 mouse stock; here too, all the correlations are positive, although the correlation for paraquat is small and does not reach statistical significance. Again, scatterplots of these relationships are shown in Figure 1 (bottom row). It is noteworthy that the size of the correlation coefficient is smallest for the isogenic CB6F1 population, and largest for the UM-HET3 mice, which have the highest level of genetic variability.

Because Scott Leiser had found that dwarf-derived fibroblasts were resistant to the metabolic effects of growth in low glucose medium, I worked in collaboration with Leiser to determine whether those cell lines most resistant to lethal stresses were also comparatively resistant to glucose reduction. Table 3.2 shows the results of a correlation analysis analogous to that shown in Table 3.1. For the genetically heterogeneous UM-HET3 mice, correlations for both peroxide and cadmium were in the range of -0.53 to -0.58, and were significant at $p < 0.006$. The negative sign indicates that those cell lines that were most resistant to cell death induced by either of these agents were also most resistant to inhibition by lowering the concentration of glucose available in media. A similarly strong, significant association was seen for the cell lines generated from Snell dwarf mice ($p < 0.007$ for both cadmium and peroxide); again those cell lines most resistant to the lethal injury of peroxide or of cadmium were those most resistant to the inhibitory effects of low glucose levels. Figure 3.2 shows illustrative scatter plots. Results for the littermate controls for the Snell dwarf mice, also shown in Table 3.2 (third line) were far less dramatic, and only reached significance for the association between glucose withdrawal and paraquat resistance ($p < 0.03$).

Discussion

The correlation data presented in Tables 3.1 and 3.2 and in Figures 3.1 and 3.2 suggest strongly that these properties may be regulated by an overlapping set of cellular mechanisms. For cell lines derived from UM-HET3 mice (11), a model chosen because of its genetic heterogeneity, there are strong and significant correlations, among individual cell lines, between peroxide resistance and resistance to paraquat, cadmium, and UV light. These correlations are also seen to some extent in cells derived from Snell dwarf, Snell dwarf normal littermate, and CB6F1 mice, with few exceptions. A correlation between resistance to peroxide and paraquat might be expected, because both agents are thought to lead to cell death by induction of oxidative damage (12;13), so cell lines with particularly high anti-oxidant defenses might be relatively resistant to both of these agents. We have previously shown, however, that UV-induced cell death in this system is not inhibited by N-acetyl-cysteine nor by Vitamin C, antioxidants that reduce the effects of oxidative damage (10). Thus, the correlation between resistance to peroxide and resistance to UV irradiation cannot easily be attributed to antioxidant defenses alone, and suggests that those cell lines with the highest levels of resistance to oxidative injury have also acquired increased protection against damage from UV light, perhaps by overexpression of genes involved in sensing or repairing DNA damage. It is interesting to note that the degree of correlation among these cytotoxic agents is highest among lines derived from genetically heterogeneous mice, and lowest among lines derived from the isogenic F1 hybrid stock CB6F1, consistent with the idea that the level of resistance may be influenced by polymorphic loci. This idea is now being assessed by a formal genetic analysis using the UM-HET3 model.

Among cell lines derived from UM-HET3 mice, those that show the greatest resistance to death induced by peroxide and cadmium are also relatively resistant to the metabolic inhibition induced by low glucose concentrations (Table 3.2, Figure 3.2). A similar relationship is seen among cell lines derived from Snell dwarf mice, although not, apparently, among cells from littermate controls of the Snell dwarf background stock. These results suggest that the metabolic pathways that modulate resistance to peroxide and cadmium, at least, overlap to some extent with those that contribute to differential

resistance to glucose scarcity. There is a significant association between resistance to glucose withdrawal and resistance to paraquat in cells from Snell dwarf littermate controls, and a similar non-significant association in Snell dwarf mice, suggesting an overlap of these pathways as well. In this context, it is noteworthy that oxidative death induced by peroxide is thought to represent a rapid induction of damage at the cell membrane very soon after contact with this short-lived agent, whereas paraquat injures cells gradually through internal production of free radicals, in particular superoxide (12;13). In contrast, there is no significant correlation between metabolic resistance to low glucose and resistance to lethality induced by UV light (not tested in UM-HET3), consistent with the idea that pathways leading to UV and glucose resistance are separate from those that contribute to resistance to the other agents tested.

There are several candidate pathways whose modulation might, in principle, affect both resistance to lethal stresses and to glucose shortage. Hexokinase, for example, the first enzyme in the glycolytic pathway, influences several elements of apoptosis, including mitochondrial release of cytochrome C (14;15), and the activity of pro-apoptotic factors (14;16;17). Glyceraldehyde-3-phosphate dehydrogenase, similarly, has been implicated in the control of DNA repair and apoptosis (18;19). Glucose withdrawal itself can lead to cell death in some cell types; the blockage of apoptosis by elevation of Bcl-x(L) indicates that in these models glucose is acting as a signal rather than solely as a nutrient (20). More generally, glucose signals modulate the activity of Akt and its downstream agonist mTOR, both of which help to regulate cell survival (21). Further, in human colon carcinoma cell lines, Akt protects from oxidative- and glucose deprivation-induced death through FOXO3a (22). Akt, JNK1, and ERK all have been shown to be involved in various models of resistance both to cell death and glucose deprivation (22-26). Similarly, both glucose withdrawal and oxidative stress have been shown to increase a number of the same proteins including CHOP and GRP-78 (27;28), mediated in part by the actions of PKCδ (29-32) using mTOR as a nutrient sensor.

It is also conceivable that co-regulated variations in sensitivity to both lethal stress and metabolic inhibition might result from differences in the function of nicotinamide nucleotide transhydrogenase (33). This enzyme catalyzes the transfer of hydrogen from NADH to NADP, a reaction coupled in the mitochondrion to the translocation of protons

from the cytoplasmic side to the matrix side of the mitochondrial membrane, and thus to the mitochondrial membrane potential. The reaction is reversible, and leads to coupled alterations in membrane potential difference and the NAD-dependent oxidation of NADPH. In effect, the transhydrogenase can make use of NAD reducing equivalents to maintain a supply of cytoplasmic NADPH. The reduced form, NADPH, plays a role in protection against lipid peroxidation and is also important for the maintenance of glutathione in its protective, reduced form GSH. Thus alterations in the level of transhydrogenase, or in its degree of coupling to mitochondrial membrane potential, might contribute to both changes in cell resistance to lethal stress and to variation in the supply of cytoplasmic or extracellular reducing equivalents under conditions of low glucose or mitochondrial inhibition.

Our working hypothesis is that fibroblasts differentiating in juvenile or young adult Snell dwarf mice are exposed to a hormonal environment that leads to long-lasting epigenetic changes in cellular traits, and that these alterations survive multiple rounds of *in vitro* cell division. Snell dwarf-derived fibroblasts are resistant to lethal oxidative and non-oxidative stresses, to DNA-alkylating agents, and, as demonstrated in this report, also to mitochondrial inhibitors and to the immediate metabolic effects of low glucose concentrations. We do not at present know which specific aspects of the hormonal milieu induce this pattern of cellular differentiation, nor how these hormonal influences produce their epigenetic changes, nor whether susceptibility to the effects of the hormones is limited to a specific window in postnatal development, nor whether similar changes can be induced in cells from normal mice by appropriate endocrine or pharmacological interventions. We also do not know what other cell types, if any, show similar patterns of stress-resistance in the dwarf animal, although we believe that multiple cell types must be affected if, as we suspect, the cellular changes contribute to the disease-resistance and long life span of these mutant mice. Further investigations of the molecular circuits that connect the metabolic effects of glucose withdrawal and mitochondrial inhibition to the other aspects of stress resistance in these cell lines is likely to provide useful insights into the pathophysiology of the unusually long-lived mice from which they are derived.

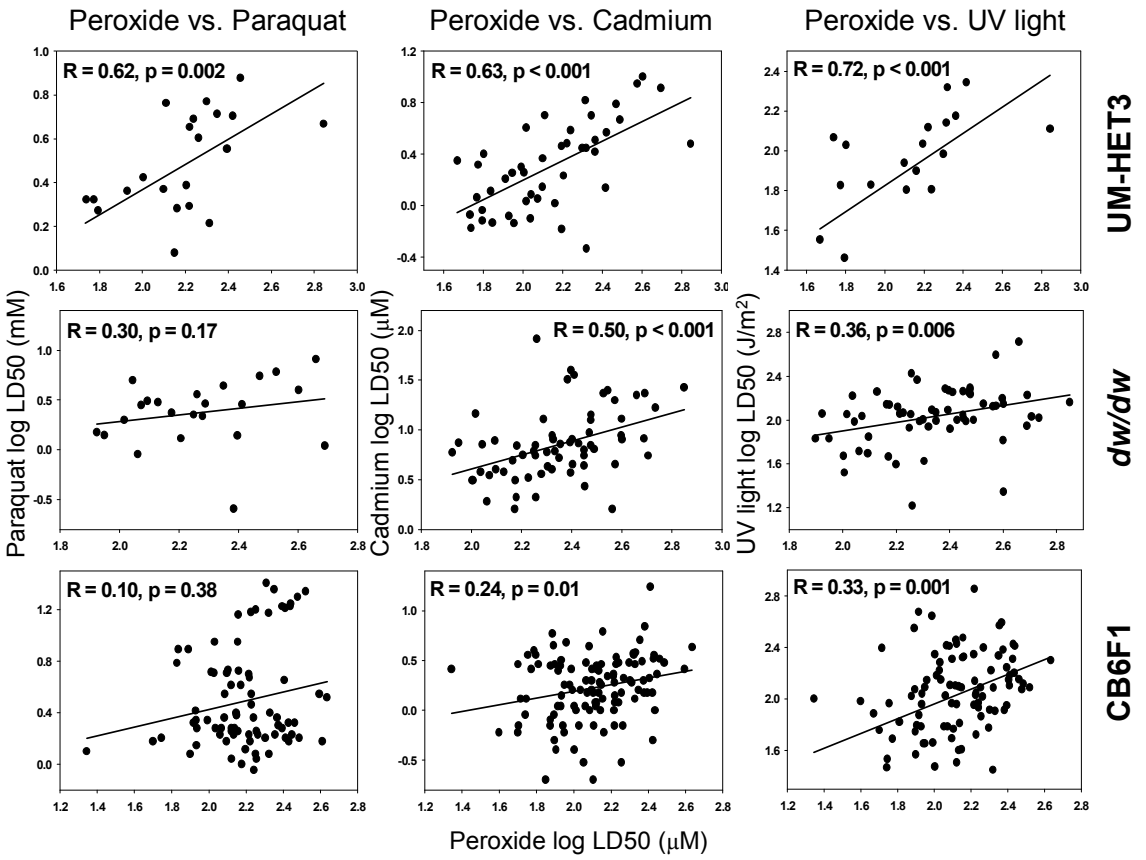

Figure 3.1. Fibroblasts isolated from UM-HET3 (top row), Snell dwarf (middle row) and CB6F1 mice (bottom row) show positive correlations between resistance to peroxide and resistance to paraquat, cadmium or UV light. The horizontal axis shows the log of the LD50 for peroxide (LD50 in μM), i.e. the dose of peroxide that led to survival of 50% of the cells. The vertical axis represents the log value of the LD50 for paraquat (in mM), cadmium (in μM), or UV light (in J/m^2). Each symbol shows LD50 values for a cell line isolated from an individual mouse. Each figure also shows the least squares regression line. R and p are the Spearman rank correlation coefficient and the significance level, respectively.

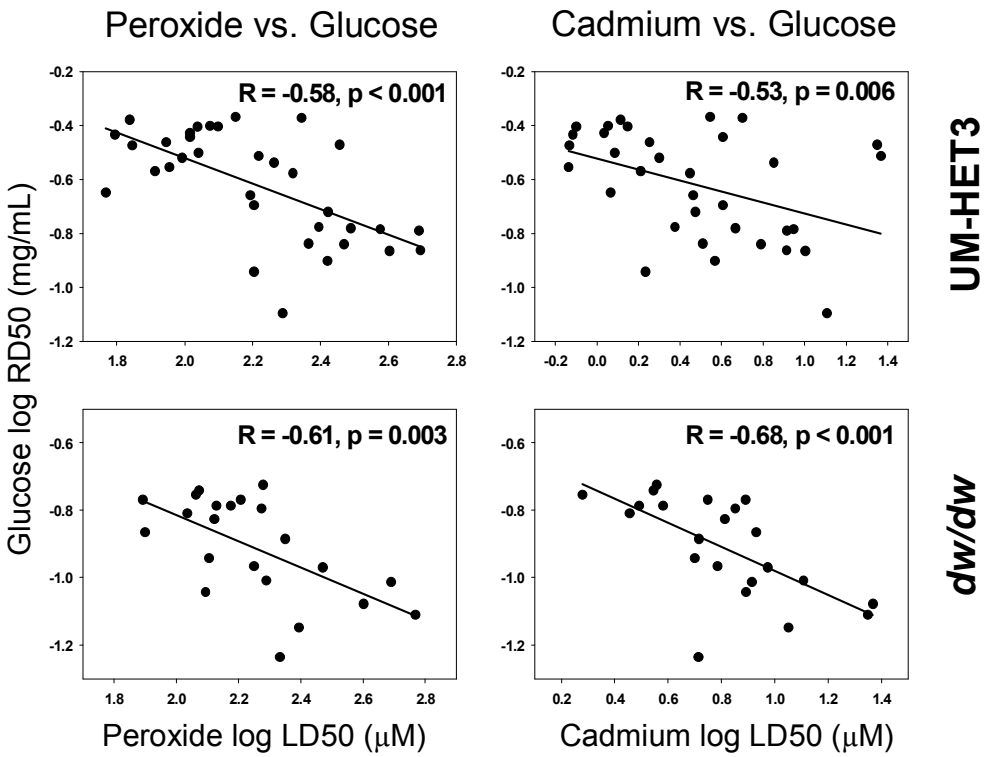

Figure 3.2. Fibroblasts from UM-HET3 (top row) and Snell dwarf mice (bottom row) show correlations between resistance to low glucose concentrations and resistance to either hydrogen peroxide (left) or cadmium (right). The horizontal axes show LD50 values for peroxide or cadmium (both in μM, on a log scale), and the vertical axis shows the concentration of glucose (in mg/ml) that leads to a 50% reduction in WST-1 conversion. Each symbol shows LD50 values for a cell line isolated from an individual mouse. Each figure also shows the least squares regression line. R and p are the Spearman rank correlation coefficient and the significance level, respectively. The negative slope of the least squares regression lines shows that mice with the highest levels of resistance to cadmium or peroxide are also most resistant to the metabolic effects of diminishing glucose concentration.

Table 3.1. Correlation of resistance to peroxide induced cell death to paraquat, cadmium, or UV light induced cell death for fibroblast lines from four mouse strains.

Strain	Correlation to peroxide [R (n)]*		
	Paraquat	Cadmium	UV light
UM-HET3	**0.62 (22)**	**0.63 (45)**	**0.72 (20)**
Snell dwarf	0.30 (23)	**0.50 (62)**	**0.36 (57)**
Snell dwarf normal littermate	**0.51 (23)**	**0.57 (61)**	-0.02 (57)
CB6F1	0.10 (81)	**0.24 (111)**	**0.33 (95)**

*R represents the Spearman rank correlation coefficient for the resistance of cells from the indicated strain to peroxide induced cell death and to cell death induced by each of the indicated stressors. The R values in bold font represent those correlations found to be significant at $p < 0.05$. The number of independent cell lines tested in each case is given as n.

Table 3.2. Correlation of resistance to peroxide or cadmium induced cell death to resistance to glucose withdrawal for fibroblast lines from three mouse strains.

Strain	Correlation to glucose [R (n)]*			
	Peroxide	Paraquat	Cadmium	UV light
UM-HET3	**-0.58 (33)**	-0.05 (8)	**-0.53 (25)**	-
Snell dwarf	**-0.44 (44)**	-0.23 (44)	**-0.41 (44)**	-0.05 (42)
Snell dwarf normal littermate	-0.05 (42)	**-0.35 (41)**	-0.12 (42)	-0.21 (38)

*R represents the Spearman rank correlation coefficient comparing the resistance of cells to glucose deprivation to cell death induced by each of the indicated stressors (peroxide, paraquat, cadmium, and UV light). The R values in bold font represent those correlations found to be significant at $p < 0.05$. The number of independent cell lines tested in each case is given as n.

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CHAPTER IV

CELLS FROM LONG-LIVED MUTANT MICE EXHIBIT ENHANCED REPAIR OF UV LESIONS

Abstract

Fibroblasts isolated from long-lived hypopituitary dwarf mice are resistant to many cell stresses, including UV light and MMS, which induce cell death by producing DNA damage. Here we report that cells from Snell dwarf mice recover more rapidly than controls from the inhibition of RNA synthesis induced by UV damage. Recovery of mRNA synthesis in particular is more rapid in dwarf cells, suggesting enhanced repair of the actively transcribing genes in dwarf-derived cells. At early timepoints, there was no difference in the repair of cyclobutane pyrimidine dimers (CPD) or 6-4 pyrimidine photophroducts (6-4PP) in the whole genome, nor was there any significant difference in the repair of UV lesions in specific genes. However, at later time points we found that more lesions had been removed from the genome of dwarf-derived cells. We have also found that cells from dwarf mice express higher levels of the nucleotide excision repair proteins XPC and CSA, suggesting a causal link to enhanced DNA repair. Overall, these data suggest a mechanism for the UV resistance of Snell dwarf-derived fibroblasts that could contribute to the delay of aging and neoplasia in these mice.

Introduction

Little is understood of the cellular mechanisms that lead to the gradual physical decline and increased susceptibility to disease that are associated with the aging process

in mammals. Mutant mice with extended life span may provide valuable resources for these studies. Snell dwarf mice have a homozygous loss-of-function mutation of the *Pit1* gene, which encodes a transcription factor regulating anterior pituitary development (1). Snell dwarf mice therefore have nearly undetectable circulating levels of growth hormone (GH) and its mediator insulin-like growth factor-I (IGF-I), thyroid stimulating hormone, and prolactin (2). These changes in the hormonal profile reduce young adult size by about 70%, extend life span approximately 40% (3) and lead to delays in many forms of pathology associated with the aging process, including arthritis, collagen cross-linking, T-cell subset changes, and the development of cataracts and glomerular lesions (3-5). These mice, as well as the phenotypically similar Ames dwarf mutants, also show a delay in the occurrence of spontaneous cancer and tend to die from cancer at a diminished rate compared to littermate controls (5;6).

Mechanisms that regulate aging may overlap with those that regulate cellular resistance to stress. Single-gene mutations that lead to an extension of lifespan in *C. elegans* tend to render those worms resistant to death induced by oxidative and non-oxidative stressors (7). To some extent, these results have been recapitulated in *Drosophila* and the mouse (8-10). For example, we have previously found that three mutations that increase life span and diminish serum IGF-I, the Ames and Snell dwarf mutations and a growth-hormone receptor KO mutation, render cells derived from these animals resistant to agents that induce cellular stress. Fibroblasts derived from Snell dwarf mice are resistant to the lethal effects of a variety of toxic agents, including oxidizing agents like paraquat and hydrogen peroxide, the heavy metal cadmium, DNA damaging agents such as UV light and methyl methanesulfonate (MMS) and incubation at 42° C (11;12). We have shown that resistance to these agents is regulated by both oxidative stress-dependent and independent pathways (12). In addition, these cells are resistant to the non-lethal metabolic effects of low-glucose media and to the mitochondrial inhibitor rotenone (13). Snell dwarf cells are also relatively resistant to growth inhibition produced by long-term culture in 20% oxygen (14).

There is increasing interest in the role of DNA damage and repair in the regulation of aging and the progression of age-related disease, particularly the development of cancer (15;16). The response to DNA damage may also be important in

the regulation of cellular stress resistance of Snell dwarf mice. In particular, they are resistant to cell death induced by UV-C light and by MMS, agents that are lethal primarily because of the lesions they induce in the cell's genome. The relationship between the formation and repair of genomic lesions and the pathways that lead to cell death are well understood. The genomic adducts formed by the energy of UV light inhibit progression of the polymerases responsible for DNA replication and transcription; these lesions lead to cell cycle arrest and apoptosis if left unrepaired (17-22). UV lesions are primarily removed by nucleotide excision repair (NER), which includes both global genome repair (GGR) of lesions in the whole genome, as well as transcription coupled repair (TCR) of lesions located in transcribed, protein-encoding genes. Cells with deficient TCR are particularly sensitive to the effects of UV radiation and undergo apoptosis at relative low doses of UV light (23). In addition to UV-induced damage, genome damage is associated with cell death in cells exposed to agents that mediate DNA damage through oxidative stress or alkylation as well (24-26).

Resistance of Snell dwarf fibroblasts to UV light-induced cell death might be caused by improved repair of DNA damage formed by irradiation. To test this idea, we used assays that measure recovery of transcription rates, and assays that measure formation and removal of DNA lesions themselves. We report here properties of Snell dwarf-derived fibroblasts that suggest an enhanced ability to repair UV-induced DNA lesions both in active genes and in the genome overall and that this enhanced repair correlates with a higher expression level of CSA and XPC which are involved in transcription-coupled repair and global genomic repair, respectively.

Methods

Animal subjects

Snell dwarf animals were *dw/dw* mice bred as the progeny of (DW/J × C3H/HeJ)-*dw/+* females and (DW/J × C3H/HeJ)F1-*dw/dw* males. These sires had been previously treated with growth hormone and thyroxine to increase body size and fertility.

Littermates with the (+/*dw*) genotype were used as controls. Skin biopsies were taken from male mice 3-4 months of age.

Cell culture

Fibroblast cultures were isolated as previously reported (11-13) in DMEM supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 0.25 μg/ml of fungizone (CM; complete media). Initial cultures of cells derived from dwarf and littermate control ("Passage 0") were grown to confluence (~95% confluence) with replacement of 2/3rds of the CM on day 3 following seeding. On day 7 following seeding, cells were subcultured by trypsinization as previously reported (12;13) and seeded at a density of 1 x 10^4 cells/cm^2 flask surface area at each passage into tissue culture flasks of 75 cm^2 surface area to produce Passage 1 cultures. Further subculturing followed this protocol. For all assays, fibroblasts were used when they had reached the end of the second passage.

Assessment of nascent total RNA synthesis following UV irradiation

Confluent, second passage cells were trypsinized and seeded into 96-well flat bottom tissue culture-treated microtiter plates in CM as described for UV resistance assays (11;12). After approximately 24 hours of incubation, cells were washed with PBS and incubated in DMEM supplemented with 2% bovine serum albumin, antibiotics and fungizone (SD medium; "serum-deprived") for approximately 24 hours. Following this period in SD media, cells were washed, then irradiated with graded doses of UV-C light (254 nm) in 100 μl warm Dulbecco's PBS (Gibco-Invitrogen, Carlsbad, CA). PBS was then aspirated and 100 μl fresh, warm SD media was added to wells. To measure total RNA synthesis, 0.5 μCi ^{3}H-uridine (MP Biomedicals, Solon, OH) was added to each well in 10 μl SD media at each time point presented. Following a 0.5 hour incubation with ^{3}H-uridine, cells were harvested with onto glass-fiber filters using 96-well FilterMate Harvester (Perkin-Elmer, Waltham, MA). CPM of incorporated ^{3}H-uridine was measured by liquid scintillation with Microscint 0 scintillation fluid (Perkin-Elmer) using

a TopCount NXT scintillation counter (Perkin-Elmer). Additionally, identically treated cultures for all stresses were assayed for cell viability using WST-1 at the same time points following irradiation. All incubations were at 37º in a humidified incubator with 5% CO_2 in air.

Assessment of nascent mRNA synthesis following UV irradiation

Confluent, second passage cells were trypsinized and 3 x 10^5 cells in 1 ml of CM were seeded into tissue culture-treated 6-well plates (Corning Costar, Lowell, MA). After approximately 24 hours of incubation, cells were washed with PBS and incubated in 1 ml warm, fresh SD for approximately 24 hours. Following this period in SD media, cells were washed and then irradiated with 10 J/m^2 UV light (254 nm) in 1 ml warm Dulbecco's PBS. PBS was then aspirated and 1 ml fresh, warm SD media was added to wells. To measure total RNA synthesis, 5 μCi ^{3}H-uridine (MP Biomedicals) was added to each well in 100 μl SD media at each time point presented. Following a 0.5 hour incubation with ^{3}H-uridine, total RNA was isolated from each well (RNeasy mini kit, Qiagen, Valencia, CA). Total DNA was isolated from parallel cultures using the DNeasy kit (Qiagen); total DNA was quantified using ND100 small volume spectrophotometer (Nanodrop, Wilmington, DE) and used to normalize RNA samples to adjust for differences in cell survival. RNA from transcribed genes (mRNA) was isolated by poly-A binding (Oligotex mRNA kit, Qiagen). mRNA was bound to glass fiber filter and the CPM of incorporated ^{3}H-uridine was measured as for total RNA above. All incubations were at 37º in a humidified incubator with 5% CO_2 in air.

Measurement of UV lesions in genome by ELISA

Confluent, second passage cells were trypsinized and seeded into dishes as for assessment of nascent mRNA synthesis above. Following 24 hours incubation in SD, cells were irradiated as above and returned to SD media. At the time points listed, and in cells that were not irradiated, genomic DNA was isolated using DNeasy tissue kit

(Qiagen) and stored in the supplied AE buffer. Genomic DNA was quantified using ND100 spectrophotometer (Nanodrop) and diluted to a stock dilution of 40 μg/mL with 1X TE buffer. ELISA plates were prepared according to the instructions from the vendor (MBL, Naka-ku Nagoya, Japan) and as based on previous publications (27;28). In brief, 96 well polyvinyl chloride sample plates (Thermo Labsystems, Franklin, MA) were prepared by coating each well with 50 μL 0.003% protamine sulfate (Sigma, St. Louis, MO) in distilled water; the plates were then dried at 37° C overnight. For measuring CPD lesions, genomic DNA was diluted to 0.4 μg/ml in 1X TE, and then denatured at 100°C for 10 minutes and chilled on ice for 15 minute. 50 μL of the denatured, diluted genomic DNA was added to each of triplicate wells for DNA isolated for each cell line at each experimental time. For measuring 6-4PP lesions, genomic DNA was diluted to 4 μg/ml and prepared similarly. Plates were then dried overnight at 37° C. For blocking, each well was washed 5X with PBS-T (0.05% Tween-20 in PBS) and then incubated with 2% FCS in 1X PBS for one hour at 37° C. Wells were then washed 5X with PBS-T and then incubated with 100 μl primary antibodies for CPD or 6-4PP (29) at a dilution of 1:2000 in PBS for 1 hour at 37° C. Wells were then washed 5X with PBS-T and incubated for 1 hour at 37° C with 150 uL Biotin-F(ab')$_2$ fragment of anti-mouse IgG (Zymed, San Franscisco, CA) diluted 1:2000 in PBS. Wells were washed 5X with PBS-T and then incubated 1 hour at 37° C with 100 μl Peroxidase-Streptavidin (Zymed) diluted 1:10000 in PBS. Following this incubation, cells were washed 5X with PBS-T and then washed once with citrate-phosphate buffer (pH 5.0). To provide quantitation, 100 μL substrate solution (o-phenylene diamene (Zymed), H_2O_2, citrate-phosphate buffer (pH 5.0) was added to each well and incubated 30 minutes at 37° C. To stop the reaction, 50 μL 2M H_2SO_4 was added and plates were read by determining the absorbance at 492 nM on SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

Quantitative PCR

Confluent, second passage cells were trypsinized and seeded into dishes as for assessment of nascent mRNA synthesis above. Following 24 hours incubation in SD,

cells were irradiated as above and returned to SD media. At the time points listed, and in cells that were not irradiated, genomic DNA was isolated using a DNeasy tissue kit (Qiagen) and stored in the supplied AE buffer. Genomic DNA was quantified using PicoGreen double stranded DNA dye (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and diluted to a final concentration of 3 ng/μL in 1X TE buffer. Quantitative PCR reactions were run as previously described with primers for the 6.5 kb fragment from 5' flanking region up to exon I of mouse DNA polymerase beta (*Polβ*) or for the 8.7 kb fragment from the 5' flanking region of mouse Beta-globin (*βglo*) (30-32). PCR reactions were run using the GenAmpXL PCR kit (Applied Biosystems, Foster City, CA) for 30 cycles in a MJ Research PTC-100 Thermocycler (MJ Research, Waltham, MA). PCR samples were quantified using PicoGreen dye according to the manufacturer's instructions.

Western Blot analysis of NER proteins

Confluent, second passage cells were trypsinized and seeded into dishes as for assessment of nascent mRNA synthesis above. Total cellular protein was isolated from cells incubated in CM for 24 hours following trypsinization and from cells that had undergone an additional 24 hours incubation in SD media. Cells were lysed in a modified RIPA buffer and stored at –20°C until analysis. Total protein content was measured by the Pierce BCA assay (BioRad, Hercules, CA). For blots, 20 μg total cellular protein extract was prepared in 6X SDS sample buffer, subjected to SDS-polyacrylamide electrophoresis and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 3% BSA, 0.1% Tween 20 in 1X PBS. CSA, XPC, XPF and XPG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1/1000 in 3% BSA, 0.1% Tween 20 in 1X PBS. Actin primary antibody (Sigma) was used at a dilution of 1/5000 in 3% BSA, 0.1% Tween 20 in 1X PBS. Alkaline phosphatase conjugated secondary antibodies (Santa Cruz) were then used at a dilution of 1/10000 in 1% BSA, 0.1% Tween 20 in 1X PBS and bands were detected using ECF reagent (GE Healthcare, Piscataway, NJ) and fluorescent scanning (Storm

System, Molecular Dynamics, Piscataway, NJ). Band intensities were measured using Image QuaNT software package (Molecular Dynamics).

Data analysis and statistics

For transcription assays, differences between dwarf and control in their uridine incorporation were analyzed separately at each time point by paired t-test. For recovery of transcription, differences between dwarf and control in the time to recovery were analyzed by the non-parametric Wilcoxon test. For ELISA assays, differences between dwarf and control DNA in the intensity of substrate signal were analyzed at each time point by paired t-test. For quantitative PCR, differences in the quantity of DNA in each amplification reaction between samples using dwarf and control DNA were analyzed at each time point by paired t-test. For Western Blots, the intensity of each bands was normalized to the corresponding β-actin and differences in these values between dwarf and control were measured under each condition by paired t-test. For each of these tests, differences were reported as statistically significant if $p < 0.05$.

Results

Fibroblasts from Snell dwarf mice are particularly resistant to the lethal effects of UV light and of MMS (11;12), agents that cause cell death primarily through the induction of DNA lesions. Because cell death induced by these agents is also associated with the inhibition of RNA synthesis, we measured transcription rates and their relative recovery in dwarf- and control-derived fibroblasts. Total RNA synthesis rates were equivalent in dwarf- and control-derived cells that had not been irradiated (Figure 4.1A). However, in cells that had been irradiated and then immediately incubated for 0.5 hours with ^{3}H-uridine, dwarf-derived cells exhibited significantly less reduction of ^{3}H-uridine incorporation for each of three doses of UV light tested, suggesting that RNA synthesis was less inhibited in fibroblasts from dwarf animals in the immediate aftermath of irradiation compared to control cells (Figure 4.1A). Such a finding might reflect either

differences in immediate DNA repair or in the ability of RNA polymerase to bypass lesions (33).

To test if there was a difference in the recovery of RNA synthesis over time, ^{3}H-uridine incorporation was measured at varying time points after UV irradiation; Figure 4.1B shows results for cells exposed to 22 J/m^2, and Figure 4.1C shows results for 45 J/m^2. These assays use the rate at which RNA synthesis recovers from inhibition as an indicator of the rate of DNA repair (19). At either of the two UV doses used, cell lines from dwarf mice showed a higher rate ofRNA synthesis at most times following irradiation compared to control cells (Figures 4.1B, 4.1C and Table 4.1). Further, we recorded, for each cell line, the time point at which recovery of RNA synthesis began after UV exposure and found that the time needed for recovery of RNA synthesis was significantly less in dwarf-derived cell lines for each of two doses of UV light (Table 4.2). It should be noted that following 22.5 J/m^2 dose of UV light, both dwarf and control cells recover RNA synthesis to levels similar to those of non-irradiated cells, whereas recovery following 45 J/m^2 UV light is not complete for either dwarf or control (Figures 4.1B and 4.1C). Higher doses of UV light led to irreversible inhibition of RNA synthesis (not shown). These results show that cells from dwarf mice recover RNA synthesis faster than control cells following UV-irradiation and suggest that repair of UV lesions may occur more rapidly in cells from dwarf animals than in control cells, at least in regions of the genome from which RNA is transcribed.

In a second series of studies, we used ELISA methods to measure two of the lesions formed in DNA by UV-C light, cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) (34). We found no difference in the background levels of CPD and 6-4PP within the genomic DNA isolated from dwarf- and control-derived cells (Figures 4.2A and 4.2B). As expected, UV light (22.5 J/m^2) induced the formation of both CPD and 6-4PP in genomic DNA isolated immediately after irradiation and there was no difference between dwarf and control cells in CPD or 6-4PP (Figure 4.2A and 4.2B). These data suggest that genomic DNA of both cell lines are equally susceptible to the energy of UV light, and thus that the difference in transcription and cell survival cannot be explained by differences in physical protection from UV. We next evaluated the time course of removal of these lesions from genomic DNA and found that there was

no difference in the rate of lesion removal between dwarf and control cell lines at early time points. However, dwarf-derived cells did show a significantly lower level of CPD lesions in DNA isolated from cells 24 hours after UV and there also a trend in this direction at 8 hours (Figure 4.2C). Similar to CPD lesions, the dwarf cells showed a significantly better removal of 6-4PP by 24 hours after UV irradiation compared to control cells (Figure 4.2D).

The cytotoxicity of UV light is closely correlated with the preferential removal of UV-induced lesions from actively transcribed, protein-encoding genes, and thus the synthesis of mRNA can be used as an indirect measure of transcription coupled repair (TCR) of these regions (19). Incorporation of ^{3}H-uridine into mRNA was measured after UV irradiation to provide an estimate of TCR. In contrast to the results for total RNA synthesis, we found no significant difference between dwarf- and control-derived cell lines in the inhibition of mRNA synthesis measured immediately after irradiation with a dose of 10 J/m^2 and 0.5 hour incubation with ^{3}H-uridine (Figure 4.3A). Nor was there any significant difference between dwarf and control in their relative mRNA synthesis when measured 2 or 4 hours following UV treatment (Figure 4.3B). However, the level of ^{3}H-uridine incorporation into mRNA in dwarf-derived cells rose rapidly between 4 and 8 hours and reached nearly double that found in control-derived cells at 8 hours after UV (Figure 4.3B). The synthesis of mRNA was also higher in dwarf-derived cells at 24 hr after irradiation, although these results did not reach statistical significance ($p < 0.06$). Together, these results show that recovery of UV-induced inhibition of mRNA synthesis occurs relatively earlier in dwarf-derived cell lines, suggesting that cells from dwarf mice have a more efficient transcription-coupled repair compared to control cells.

To directly test whether UV-lesions in actively transcribed genes are removed faster in dwarf cells compared to control cells, we used quantitative PCR (QPCR) to assess the levels of lesions at different times after UV-irradiation in two specific genes, *Polβ* and *βglo*. The assay measures the extent to which DNA lesions prevent the progress of DNA polymerase in a PCR amplification of the specific DNA sequences of interest; amplification of DNA containing unrepaired lesions is expected to be less than that of non-irradiated or repaired DNA (30-32). In unirradiated cells, we found no difference between dwarf and control in the amount of amplification of a region of *Polβ* (Figure

4.4A), which is actively transcribed in fibroblasts, or in the amount of amplification of a region of *βglo* (Figure 4.4B), a gene that is not transcribed in fibroblasts (a negative control). Compared to non-irradiated cells, *Polβ* and *βglo* amplification was inhibited approximately 50%, in both dwarf and controls cells, when using genomic DNA isolated immediately after 10 J/m^2 UV light (Figures 4.4A and 4.4B). Amplification of *Polβ* increased with time after irradiation indicating that UV lesions were being removed from these sequences (Figure 4.4C). Amplification of *Polβ* tended to be higher in dwarf-derived cells over time, although the results did not reach statistical significance. For *βglo*, we found no evidence for any change in relative amplification over time and no difference between dwarf and control suggesting that lesions were not being removed from this non-transcribed gene fragment (Figure 4.4D).

Seeking molecular explanations for the differences between dwarf and control cells in the repair of UV-induced damage, we used immunoblotting to measure the protein levels of some of the enzymes important in nucleotide excision repair (NER). XPC is the DNA binding subunit of the XPC-hHR23B complex (35) and has affinity for multiple sites of genomic damage, in particular bulky adducts like 6-4PP that introduce helical distortions in the nucleic acids (36-39). Recognition of lesions repaired by TCR occurs by recruitment of the CSA and CSB proteins to the site of the stalled RNAPII complex (19;40;41). XPF and XPG are endonucleases that incise on the 3' and 5' side of the damaged DNA region respectively (42;43). Lysates were prepared in two conditions: (a) from cells grown to confluence in complete medium and (b) confluent cells that had been further incubated for 24 hr in medium without serum. This serum-free condition was evaluated because the difference in stress resistance between dwarf and normal cells is most pronounced under these circumstances (11), and because lysates from serum-deprived cells had been used for the DNA repair assays presented above. We saw no difference in the expression of any of four NER proteins using lysates from cells grown to confluence in medium containing serum (Figure 4.5). In cells that had been incubated in serum-deprived media, however, we found significantly more XPC and CSA expression in dwarf-derived cell lines compared to control cells. There was no difference between serum-deprived dwarf and control cells in their expression of XPG or XPF.

Discussion

The energy of UV light is absorbed by DNA and causes the formation of bulky genomic lesions in DNA. These lesions play a significant role in cytotoxicity, mutagenesis, and the development of neoplasia in mammals (44;45). Therefore, the rapid and efficient repair of these lesions is of critical importance to cell survival and tumor suppression (15). Further, differences in the resistance to UV-induced cell death might result from differential response and repair of UV-induced DNA lesions (23;46). We have previously found that skin-derived fibroblasts from the long-lived Snell dwarf mice, and related long-lived mouse models, are resistant to the cytotoxic effects of UV-light (11;12). Here we report that the enhanced survival of dwarf-derived fibroblasts is accompanied by an enhanced ability to repair UV lesions both within active genes and within the genome as a whole and an enhanced recovery of mRNA synthesis following UV irradiation. Further, we find that differences in DNA repair are associated with expression levels of NER proteins thought to play roles in the cellular recognition of blocked RNA polymerase II complexes as well as global genomic DNA lesions.

Cell death caused by UV light is, at least in part, due to the cytotoxicity of bulky UV-induced lesions formed within actively transcribing genes; these adducts block the progression of RNA polymerase and inhibit transcription (18;23). Cells from donors with TCR defects, such as those from patients with Cockayne's Syndrome (CS) or Xeroderma Pigmentosa complementation group A (XP-A), have poor recovery of mRNA synthesis following UV and undergo apoptosis at relatively low doses of UV radiation (23;47). In addition, cells deficient in pRb, a protein thought to play a role in GGR, also show UV sensitivity and poor recovery of mRNA synthesis following UV irradiation (46;48). These data suggest that UV lesion repair regulates the cell's sensitivity to irradiation and hint at a potential mechanism for the resistance of Snell dwarf cells to DNA damaging agents. The relatively rapid recovery of mRNA synthesis after UV irradiation (Figure 4.3B), an index of TCR, provides a plausible explanation for the resistance of Snell dwarf cells to death induced by UV. Our QPCR data on repair of UV-induced lesions in *Polβ*, an actively transcribed gene, are consistent with this idea, although the differences between normal and dwarf mice were not statistically significant

in this assay. Since this assay measures removal of lesions from both strands of the sequence to be amplified, removal specifically from the transcribed strand will be somewhat obscured, and therefore, this assay will underestimate TCR.

We found relatively little removal of UV-induced CPD and 6-4PP, though there were significantly fewer CPD and 6-4PP in DNA from dwarf cells 24 hours after irradiation. This relatively modest level of repair is consistent with that reported for other groups using rodent-derived cell lines (49-51) and presumably reflects the well-documented deficiency of GGR in rodents (52). The low level of GGR is consistent with our QPCR data on *βglo,* a gene that is not transcribed in fibroblasts: we see no recovery in amplification of *βglo* DNA in the 24 hours following irradiation, suggesting that no UV lesions have been repaired. However, a region of a transcribed gene, *Polβ*, was repaired effectively, with amplification recovering slowly in the 24 hours after irradiation. Amplification of *Polβ* sequences returned to 90% (± 13% SEM) of non-irradiated levels in dwarf cells, compared to 72% (± 18% SEM) in control cells at 24 hr, suggesting that more lesions had been repaired in dwarf-derived cells, although the difference is not statistically significant.

We found no evidence for a difference between dwarf and control cells in the formation of UV damage immediately after irradiation within either the whole genome as measured by ELISA or in specific regions as measured by QPCR. There was also no difference in the synthesis of mRNA measured at the earliest time point, 0.5 hours following irradiation. These observations are all consistent with the working hypothesis that DNA in dwarf and control cells is equally vulnerable to the immediate effects of UV exposure. In contrast, we did note that transcription of total RNA was significantly higher in dwarf-derived cells when measured in the 30 min immediately after UV exposure. This disparity raises the possibility that polymerases for non-mRNA transcripts may be able to bypass UV lesions to differing degrees in dwarf and control cells. There is little data on RNAPI or RNAPIII, but in Chinese hamster ovary cells and in human cells recovery of RNA synthesis has been shown to precede the strand-specific removal of photolesions from the genome, presumably because RNAPII is able to bypass the remaining lesions (33;53). Alternatively, it seems possible that some portion of the DNA damage is repaired so rapidly after irradiation that differences between dwarf and

controls cells can be observed even within the 30 min incubation needed to measure the rate of ^{3}H-uridine incorporation.

Our data suggest that the onset of repair following irradiation occurs more rapidly in cells from Snell dwarf mice. The cellular recognition of lesions within the genome has been proposed as the rate-limiting step of NER (54) in part because of the large number of bases in the genome that must be scanned for many types of damage and also because of the physical limitations of the sequential recruitment of repair proteins to the site of damage (55). Cells expressing more of the NER proteins required for recognition of UV lesions may thus be able to repair lesions more rapidly than those expressing proteins at lower levels. We found that dwarf-derived cells expressed higher levels of the proteins XPC and CSA than control cells, when evaluated after 24 hr incubation in serum-free medium. The binding of XPC to UV lesions is thought to guide the formation of the NER complex for the execution of GGR (56-58). CSA and CSB bind in concert to the stalled RNAPII and facilitate the assembly of the TCR complex, thereby acting as the de facto recognition proteins for TCR (59-62). It has also been proposed that CSA protects the stalled RNAPII and CSB from proteasomeal degradation (40;63). Higher levels of CSA may thus stabilize the intact TCR complex leading to more efficient transcription coupled repair. Interestingly, there was no difference between dwarf and control in the expression of the NER excision proteins XPF and XPG (42;43) suggesting that differences in DNA repair might be mediated by differences in the level of proteins involved in the rate limiting steps of repair and not necessarily up-regulation of the entire pathway. It would be of interest to explore if overexpression of these proteins in cell lines, or in transgenic mouse models, alters cellular stress resistance and organismic longevity.

Dwarf-derived cells were found to repair UV lesions more rapidly both within the whole genome, and to some extent, in transcribed regions. Unrepaired UV lesions located within transcribed genes can cause cell death (17-20;23) and are thus plausible explanations for differential resistance to UV-induced cell death. Lesions in the 95% of the genome that is not transcribed in a given cell type are considered primarily mutagenic and are of little consequence to survival of the cell in the short term (19). The difference in global repair of CPD and 6-4PP presented in Figure 3.2 may contribute to the

comparatively low incidence and the delay of neoplasia in dwarf mice (5;64). The contributions of NER deficits to deficiency in DNA repair are well documented, but less is known about the control of DNA fidelity over the long-term, and the balance between cell death and transformation that modulates incidence and progression of neoplastic lesions. There are some limited data suggesting that Snell dwarf mice are resistant to DNA damage, in vivo; treatment with X-ray or mitomycin C results in fewer mutagenic lesions in erythrocytes, bone marrow cells and spermatogonia of Snell dwarf mice (65;66). Data on the formation and repair of CPD and 6-4PP in the DNA of skin cells of irradiated dwarf and control mice would provide a useful in vivo correlate for our in vitro observations. There is also some suggestion that the development of neoplasia induced by mutagens and implanted tumors is diminished in Snell dwarf mice (67-70). Therefore, it would be interest to test if Snell dwarf mouse were resistant to the development of cancer induced by an assortment of agents that induce repairable DNA damage, including UV light.

Many have speculated that the loss of genome integrity may contribute to aging, and that differences in DNA repair processes might modulate the rate of aging per se (71;72). Age-related decline has been observed in the ability of many species to repair UV damage as measured by unscheduled DNA synthesis (UDS), including human and mouse (73-76). More interestingly, a number of studies have suggested that the repair of UV lesions is correlated with longevity across mammals (77-79). However, our own data and that of others suggest that there is much variation among rodent species of different longevities in their response to UV damage (80;81). Caloric restriction, which extends lifespan in most tested species (82), has also been shown to improve repair of DNA after UV-irradiation. For example, dietary restriction enhances UV repair of kidney cells in rats (83), hepatocytes in rats and mice (75;83), fibroblasts derived from the lung and the skin of rats (84;85), and splenocytes in mouse (86). While these studies measured GGR, it has been shown that the removal of CPD lesions from specific genes is enhanced in the hepatocytes of calorie restricted rats, suggesting enhanced TCR (87).

It is not clear how a better system of UV-lesion repair may directly contribute to the extended lifespan of dietary restricted animals or of the Snell dwarf mouse reported here. The large number of cell types showing greater repair capacity in calorie restricted

models suggest global protection from damage. It may be that enhanced DNA repair contributes to the decreased accumulation of DNA damage in long-lived animals which could lead to diminished cell senescence (88), diminished cell loss by apoptosis (89), diminished p53 induction (90), or diminished development of neoplastic cells (15). In contrast, some evidence has been presented that suggests that high levels of global damage do not adversely affect longevity or age-related physiological decline. For instance, mice lacking the mismatch repair gene *Pms2* have high mutation frequencies, but no change in lifespan and only a slight increase in carcinogenesis (91). Similarly, mice lacking the antioxidant superoxide dismutase (MnSOD) show high levels of oxidative lesions to both nuclear and mitochondrial DNA, have higher incidences of multiple tumor types, but have the same lifespan as their controls (92). Lastly, the maximum life-span of naked mole-rats is approximately 8 times longer than mice, and these animals seem to be essentially free of spontaneous late-life cancer, and yet their tissues show significantly more DNA oxidation than those in mice (93). It may well be the connections between DNA repair and longevity are somewhat indirect and limited to some shared mechanisms of the repair of particular types or locations of DNA damage.

This study suggests mechanisms that may help explain the longevity and low cancer incidence in hypopituitary dwarf mice. By expressing a higher level of CSB protein involved in transcription-coupled repair, dwarf cells remove cytotoxic transcription-blocking lesions faster than cells from control mice, leading to a faster recovery of mRNA synthesis after insult. Under some circumstances, faster repair might protect cells from apoptosis, and thus preserve function of tissues in which cell replacement is limiting. Dwarf cells also express high levels of XPC which enhances the removal of mutagenic lesions by GGR; removal of these lesions might contribute to the low cancer incidence of dwarf mice (5;94). By enhancing both TCR and GGR without losing the balance between these repair pathways, dwarf mice enjoy longevity without paying the price of increased cancer incidence. Much remains to be learned, however, about the ways in which the endocrine abnormalities of the Snell dwarf mouse lead to long-lasting improvements in DNA repair pathways, about how these pathways are coupled to improved resistance to other forms of lethal and metabolic stress (11-13), and

about the extent to which similar stress resistance might alter the properties of other cell types within the dwarf mouse.

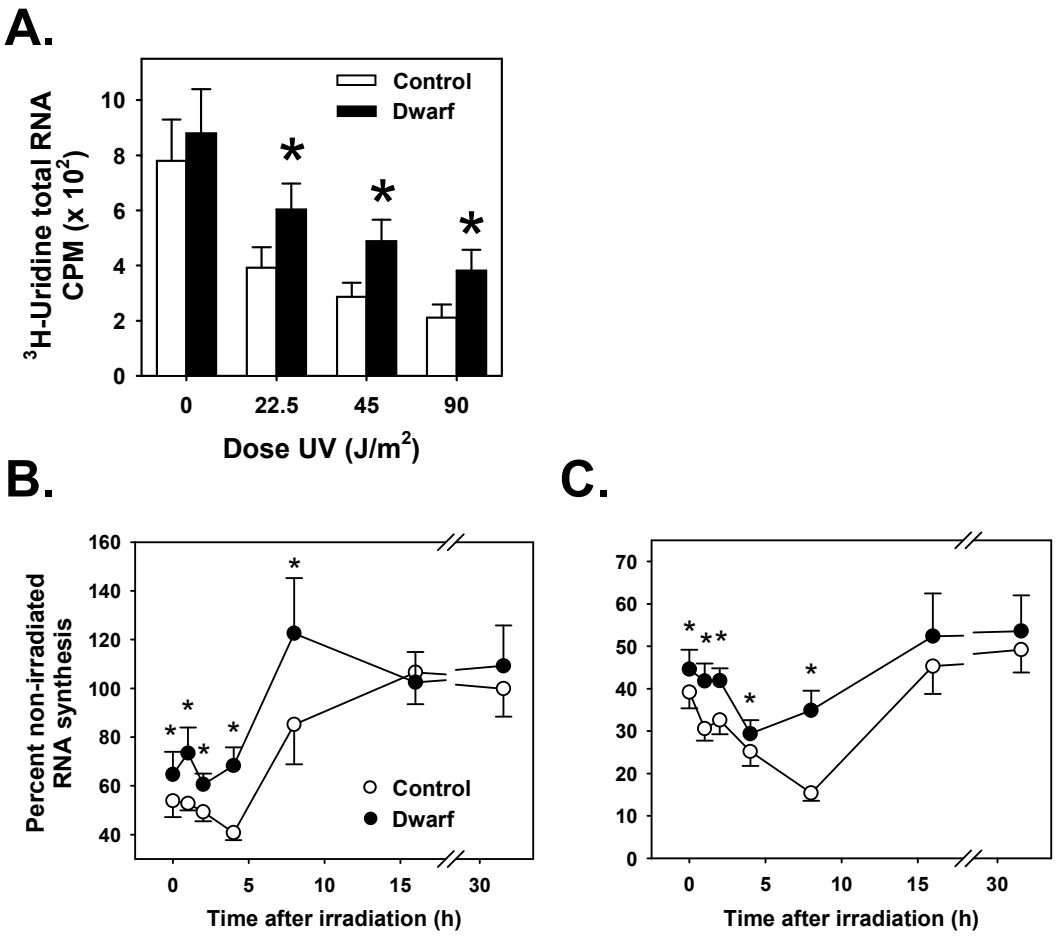

Figure 4.1. Fibroblasts from Snell dwarf mice show less inhibition of total RNA synthesis following UV irradiation than fibroblasts from control mice. (A.) Cell lines isolated from 9 individual Snell dwarf mice (filled bars) and 9 individual controls (open bars) were assayed for ^{3}H-uridine incorporation into total RNA as a function of dose of UV irradiation. Cells were irradiated then incubated with ^{3}H-uridine for 0.5 hour prior to total RNA collection. Error bars represent SEM and * represents $p < 0.05$ for paired t-test between genotypes for each individual dose of UV light. (B.) Each circle represents the average ^{3}H-uridine incorporation of UV-irradiated (22.5 J/m^2) cell lines from 9 Snell dwarf mice (filled circles) and from 9 controls (open circles) relative to their respective non-irradiated controls. Cells were irradiated and then incubated in SD media for times indicated on X-axis prior to total RNA collection. Error bars represent SEM a * represents $p < 0.05$ for paired t-test between genotypes for each individual dose of UV light. (C.) As in B., for 45 J/m^2 dose of UV light.

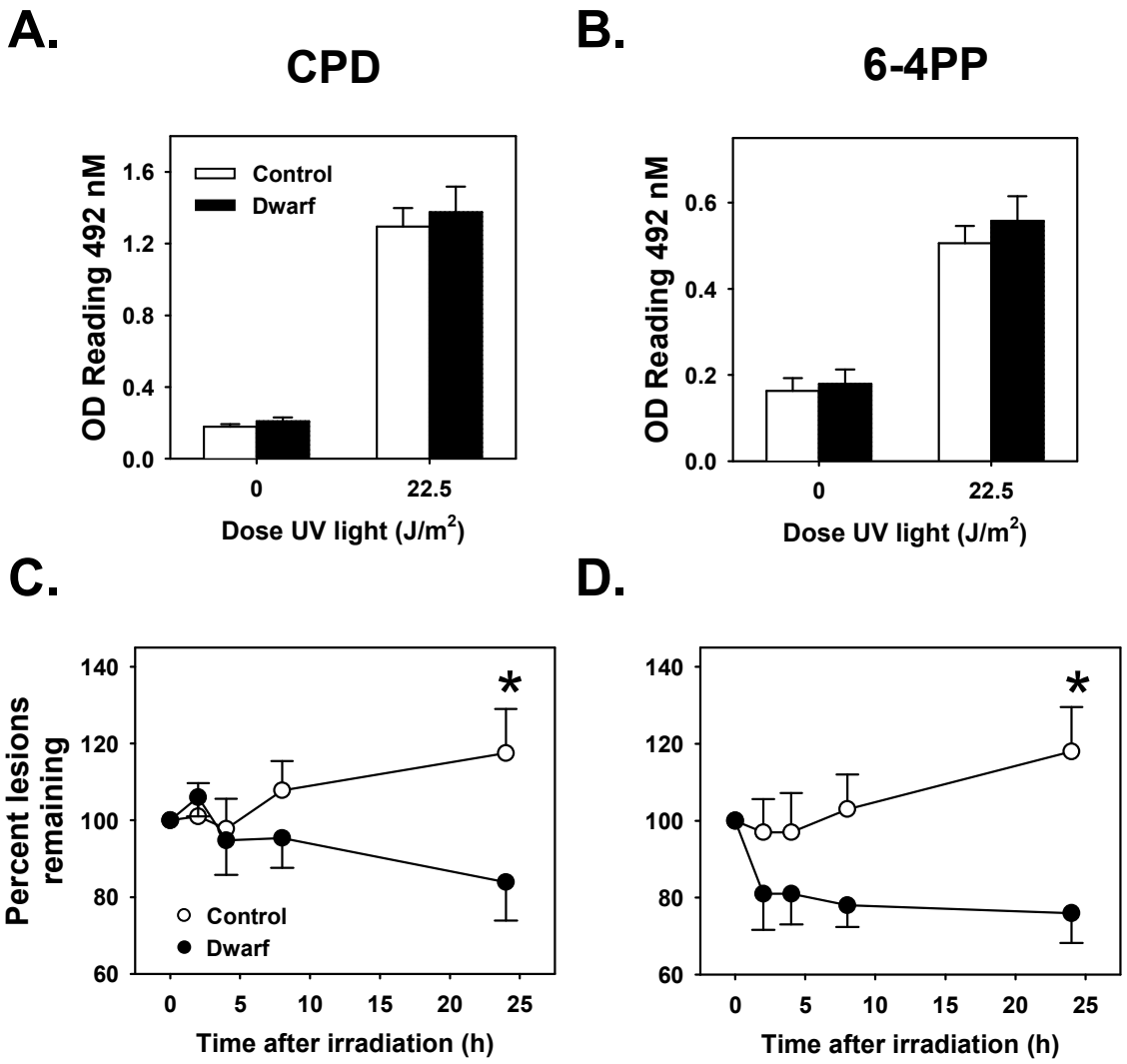

Figure 4.2. Fibroblasts from Snell dwarf mice remove significantly more CPDs and 6-4PPs from the whole genome over time compared to control cells. (A.) Bars represent the average value quantified by spectrophotometer of the presence of CPD lesions in genomic DNA from cell lines isolated from 11 individual Snell dwarf mice (filled bars) and 11 individual controls (open bars). Genomic DNA was isolated from non-irradiated cells or immediately following irradiation as indicated by X-axis. Error bars represent SEM. (B.) Presence of 6-4PP presented as in A. (C.) Circles represent the average CPD ELISA result for genomic DNA isolated from cells at the indicated times following irradiation, relative to the results presented in A. Closed circles represent the results from cell lines derived from 11 different Snell dwarf mice and open circles represent those from 11 controls. Error bars represent SEM and * represents $p < 0.05$ for paired t-test between genotypes at that time point. (D.) Presence of 6-4PP presented as in C.

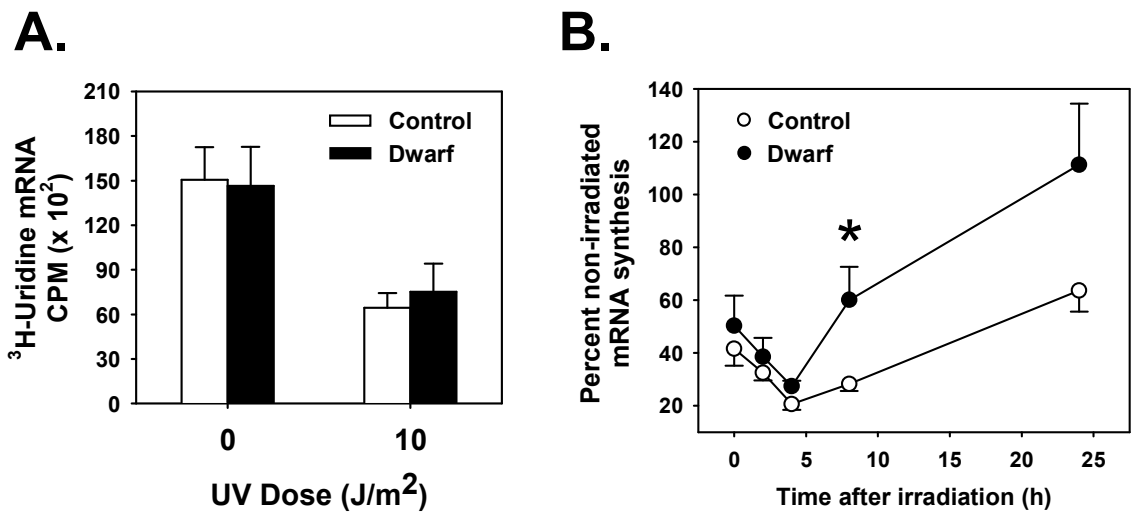

Figure 4.3. Faster recovery of mRNA synthesis following UV-irradiation in fibroblasts from Snell dwarf compared to control cells. (A.) Cell lines isolated from 6 individual Snell dwarf mice (filled bars) and 6 individual controls (open bars) were assayed for ^{3}H-uridine incorporation into mRNA following UV irradiation. Cells were irradiated (or not) then incubated with ^{3}H-uridine for 0.5 hour prior to mRNA collection. Error bars represent SEM. (B.) Each circle represents the average ^{3}H-uridine incorporation of UV-irradiated cell lines from 6 Snell dwarf mice (filled circles) and from 6 controls (open circles) relative to their respective non-irradiated controls. Cells were irradiated and then incubated in SD media for time indicated on X-axis prior to total RNA collection. Error bars represent SEM a * represents $p < 0.05$ for paired t-test between genotypes for each individual dose UV.

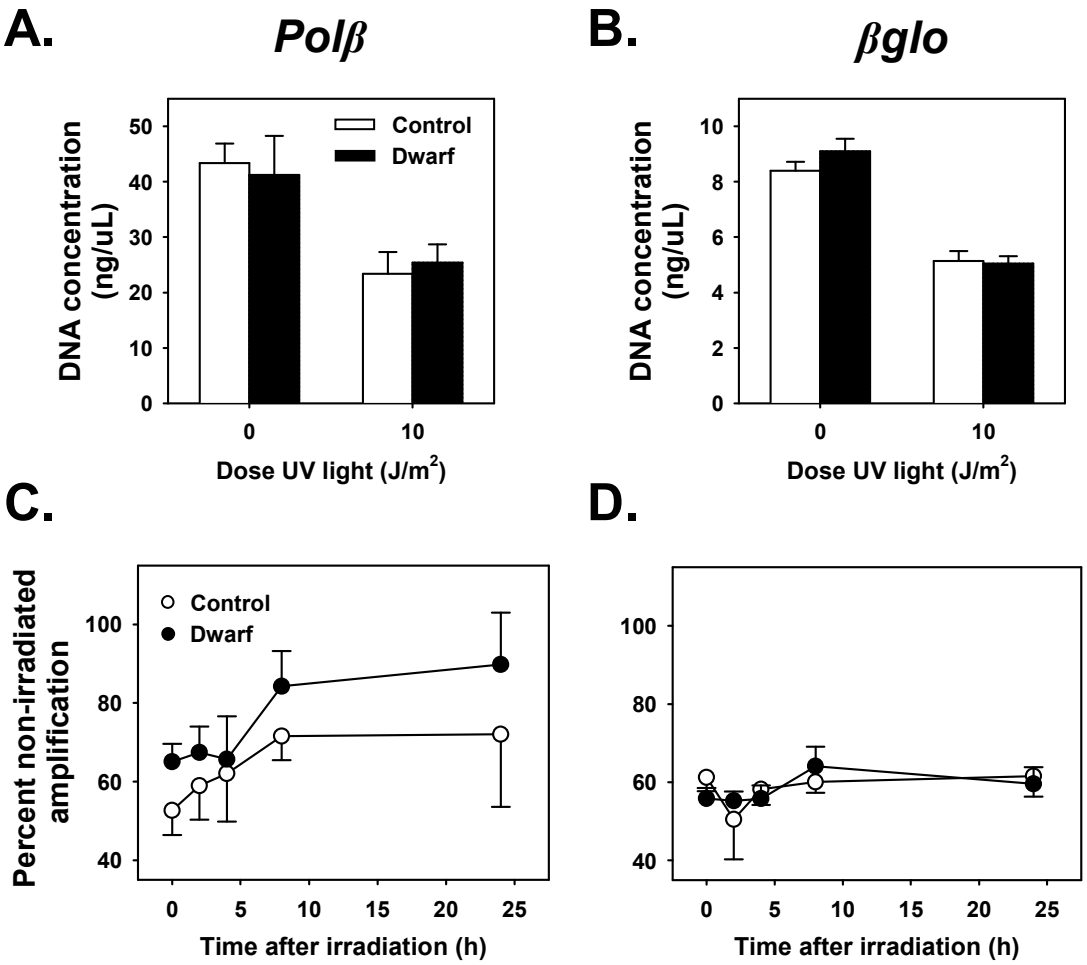

Figure 4.4. There is no significant difference in the formation or removal of UV lesions from two specific genes as measured by amplification of QPCR products using genomic DNA isolated from irradiated cells. (A.) Bars represent the average concentration of DNA measured following QPCR of *Polβ* using genomic DNA from non-irradiated cells and genomic DNA isolated immediately following UV irradiation (X-axis). Bars represent the average results from the genomic DNA of cell lines isolated from 6 individual Snell dwarf mice (filled bars) and 6 individual control mice (open bars). Error bars represent SEM. (B.) As in A., for QPCR of *βglo*. (C.) Each circle represents the average concentration of QPCR products for *Polβ* using genomic DNA from cells irradiated and then incubated in SD media for time indicated on X-axis prior to isolation of DNA. Filled circles represent the results of genomic DNA from cell lines isolated from 6 Snell dwarf mice and open circles represent those from 6 control mice. Error bars represent SEM. (D.) As in C., for QPCR of *βglo.*

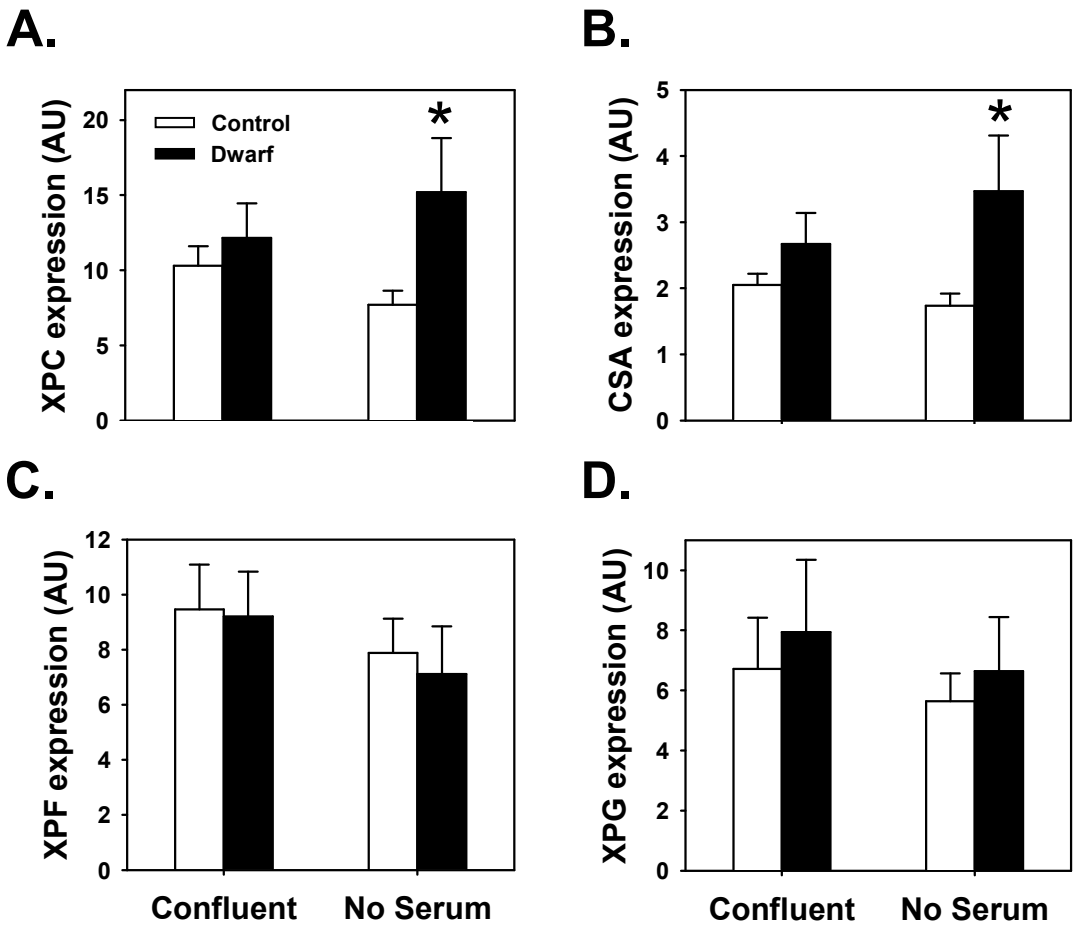

Figure 4.5. Fibroblasts from Snell dwarf mice express higher levels of XPC and CSA following serum deprivation compared to control cells. For each graph, the X-axis represents the cell growth condition from which total cell protein lysates were made. Bars represent the average expression of the indicated protein as measured by immunoblot of 20 μg protein from total cell lysates and normalized to β-actin expression. Filled bars represent the average expression of the indicated protein in cell lines isolated from 7 individual Snell dwarf mice and open bars represent the average expression in cell lines isolated from 7 control mice. Error bars represent SEM and * represents $p < 0.05$ for paired t-test between genotypes for that particular treatment. (A.) The cumulative results from immunoblots for XPC. (B.) The cumulative results from immunoblots for CSA. (C.) The cumulative results from immunoblots for XPF. (D.) The cumulative results from immunoblots for XPG.

Table 4.1. ^{3}H-uridine incorporation following UV irradiation is greater in dwarf-derived fibroblasts.

	Average percent control CPM of ^{3}H-uridine uptake, normalized by experiment day (SE)					
Time following UV (hours)	**22.5 J/m^2 UV light**			**45 J/m^2 UV light**		
	Control	Dwarf	Paired t-test p	Control	Dwarf	Paired t-test p
No UV	100 (9)	112 (14)	0.24	100 (9)	112 (14)	0.24
0	100 (11)	171 (23)	**0.005**	100 (12)	174 (15)	**0.001**
0.5	100 (9)	140 (14)	**0.02**	100 (9)	135 (12)	**0.03**
1	100 (10)	120 (8)	**0.05**	100 (10)	142 (20)	**0.04**
2	100 (8)	157 (24)	**0.02**	100 (11)	152 (16)	**0.01**
4	100 (10)	226 (14)	**<0.001**	100 (14)	142 (18)	**0.04**
8	100 (8)	151 (14)	**0.01**	100 (10)	217 (29)	**0.002**
16	100 (6)	112 (8)	0.16	100 (8)	133 (23)	0.08
32	100 (5)	131 (17)	0.08	100 (8)	90 (20)	0.34

For each time point, cells lines from 9 individual animals of each genotype were assayed for ^{3}H-uridine incorporation following UV irradiation. An average value for control cells at each time point was calculated and both dwarf and control values were normalized to this. P values in bold represent significance at $p<0.05$.

Table 4.2. Inhibition of transcription is repaired more rapidly in Snell dwarf-derived fibroblasts

UV dose	Genotype	Sample size	Average Time to Recovery (hr)	SE	Wilcoxon (p)
22.5 J/m^2					
	Snell dwarf	9	5.3	0.8	
	Control	9	8.9	1.2	p = 0.017
45 J/m^2					
	Snell dwarf	9	10.7	1.4	
	Control	9	19.6	2.4	p = 0.011

Average time to recovery represents the time point at which RNA synthesis begins has reached its minimum level, relative to unirradiated cells, and the levels of uridine incorporation begin to rise. Wilcoxon (p) represents the p value calculated for a Wilcoxon non-parametric test.

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CHAPTER V

LONGEVITY AND STRESS RESISTANCE ACROSS MAMMALIAN SPECIES: SKIN-DERIVED FIBROBLASTS FROM LONG-LIVED SPECIES ARE RESISTANT TO SOME, BUT NOT ALL, LETHAL STRESSES AND TO THE MITOCHONDRIAL INHIBITOR ROTENONE

Note: Much of this material is excerpt from an article by James M. Harper, Adam B. Salmon, Scott F. Leiser, Andrezj T. Galecki, and Richard A. Miller published in Aging Cell. 2007 Feb;6(1):1-13. For this work, I generated the cell lines from all of the species tested. In addition, I also generated the stress resistance data for all of the lethal stressors, and the data for glucose and rotenone were generated by work by myself and Scott Leiser. I performed the initial regression analysis comparing cellular stress resistance and maximum lifespan of the donor; all other stastical analyses were performed by James Harper, Andrezj Galecki, Sue Chen and Richard Miller. For the journal article, I generated the initial figures for publication, as well as portions of the methods and discussion write-up.

Introduction

The rationale, pitfalls, and background for using a comparative biology approach to explore mechanisms of aging have been discussed in Chapter I of this thesis. In brief, the differences in longevity across different mammalian species are much greater than those that can be created through single-gene mutations or dietary restriction (1). However, it is unknown if the mechanisms that generate long lifespan through species evolution are similar to the mechanisms by which lifespan is extended in genetic mutants or dietary interventions using a single species.

In previous chapters of this thesis, I have shown that fibroblasts derived from long-lived Ames and Snell dwarf mice and growth hormone receptor knockout (GHR-KO) mice are resistant to the lethal effects of many types of cellular stressors, both oxidative and non-oxidative. Scott Leiser, another graduate student in the lab, has shown that cells from Snell dwarf mice are also resistant to non-lethal effects of incubation in low-glucose media or in the presence of the mitochondrial poison rotenone. Thus, these data suggest a model in which hormonal abnormalities in Snell dwarf mice, and presumably Ames dwarf and GHR-KO mice, might induce both metabolic alterations and stress resistance in multiple cell types, some of which may contribute to the delayed progression of multiple forms of late-life illnesses and slowed aging in these animals.

To see if skin derived fibroblast cells from long-lived mammals might resemble cells from dwarf mice in their resistance to multiple lethal and non-lethal agents, we developed a panel of cells from multiple adult donors of eight species of wild-trapped rodents (Table 5.1). We also included cells derived from a stock of laboratory-raised ("DC") mice whose genes derive from four commonly used domesticated inbred lines; this was in part due to previous observations that wild-derived mice differed from laboratory-adapted mouse stocks in size, hormone levels, maturation rate, and longevity (2). Lastly, we included multiple cell lines from the little brown bat (*Myotis lucifugus*), which is much longer lived than rodents of similar body weight. Following data collection, the results were analyzed using different statistical methods that attempt to address some of the specific pitfalls of using samples from across a number of different mammalian species. Our data suggest that longevity, in this collection of species, is associated with cellular resistance to the lethal effects of cadmium, hydrogen peroxide, and heat, and possibly with resistance to MMS as well, in addition to resistance to the metabolic effects of mitochondrial inhibition. In contrast to the data on Snell dwarf mice, we saw no evidence across species for association of longer lifespan to resistance to UV light or paraquat.

Results and Discussion

Cell lines derived from each species, with lab-derived and wild mice being treated separately, were tested in vitro for their resistance to six varieties of lethal stress, as well as for resistance to the non-lethal metabolic effects of the mitochondrial inhibitor rotenone and of culture at very low glucose levels. Figure 5.1 presents, for each of the six lethal stresses tested, scatterplots of mean LD50 values against the life span estimate for each of the nine kinds of animals listed in Table 5.1, treating both *M. musculus* groups separately. The bars show standard errors of the mean for each LD50 value, except for beaver (for which only a single individual donor was tested.) The regression values (Pearson R^2, and associated p-value) refer to a standard linear regression model in which each species contributed a single, average value for its LD50, with no adjustment for phylogeny, body weight, or number of individuals tested per species. Using this method of analysis, maximum life span is positively correlated with fibroblast cell line resistance to cadmium and hydrogen peroxide, and regressions for MMS and heat are not significant, but yield $p < 0.08$ in each case, suggesting a trend for association between life span and resistance to these two stressors. There is no indication for any relationship between life span and resistance to UV light or to paraquat. Because fibroblasts from Snell dwarf mice are also resistant to the metabolic effects of the mitochondrial inhibitor rotenone and of low-glucose culture media (3), we assayed this collection of fibroblast cell lines for their response to these agents. These results are presented in Figure 5.2, and show that cells from long-lived species are relatively resistant to the inhibitory effects of rotenone. These results also suggest that there is a trend for association between lifespan and resistance to the effects of glucose withdrawal with $p = 0.09$.

Table 5.2 presents the results of the standard linear regression as well as secondary analyses performed on these data by my colleagues James Harper, Andrzej Galecki, and Shu Chen. The multi-level regression was used to test the conclusions of our initial results for robustness to the arbitrary design and calculates an "across-species" variability term that is separated from the "between-species" variation. The p-value then represents the statistical significance of the regression of the "across-species" term. This analysis, like the standard linear regression, suggests significant effects for cadmium,

peroxide, and rotenone across this group of species; in addition, the regression for heat stress, for which $p = 0.053$ in the standard regression, is significant in the two-level regression at $p = 0.02$. The third column of Table 5.2 presents the results of a statistical approach that evaluates the data with an added term that accounts for phylogetic relatedness of the species tested. These results show that the association of cadmium and peroxide resistance with species longevity remains significant after adjustment in this way; the rotenone association is no longer significant.

The same regression analyses were also performed using a subset of the data limited to wild-trapped rodents only, thereby eliminating potential effects of the non-rodent (bat) species and of the laboratory-adapted mice. These results are presented in Table 5.3. Among this set of species there is a significant association between longevity and glucose resistance, for each of the three regression methods. In this smaller data set, however, some of the associations seen in Table 5.2 are no longer statistically significant, suggesting that they may have been driven by one of the particular species tested in this small group.

As discussed in Chapter I, one major pitfall of comparative biology is that regressions of molecular and cellular parameters on longevity may reflect the trend toward long-life among larger mammals (4;5). Thus, our data set was also analyzed by James Harper with weight-adjusted lifespan of each species for lifespan that were generated using residuals, or estimates of how much the observed lifespan differs from the expected lifespan based on body weight. Table 5.4 shows the outcome of two such adjusted regression studies, with the first column showing regressions in which the average resistance of cell lines from each species was regressed against the lifespan residual. This analysis showed significant associations for cadmium, peroxide, heat, and rotenone in this set of models. In the second column, the weight-adjusted lifespan was compared to a weight-adjusted resistance value, which was calculated similarly to the lifespan residual. This method of analysis also showed significant associations for cadmium, peroxide, heat, and rotenone.

These results have several implications for the mechanisms that may regulate aging across mammalian species. Despite the small number of species and individuals examined, we found an association between longevity and cadmium and hydrogen

peroxide resistance that is robust across several different analytical methods, and also show varying degrees of support for parallel hypotheses for rotenone, heat, and perhaps MMS. Most of these associations remained significant even after an analytical approach that adjusts for the effects of average species weight. Similarly, these associations also tend to remain even when analyzed by statistical approaches that are designed to eliminate effects of phylogenetic relatedness. It therefore seems unlikely that that the differences in resistance among species tested in this data set only reflect variation in species body size or in the degree of relatedness among the species tested. Thus, these results suggest that the evolution of long lifespan among mammalian species has, similarly to lifespan extending mutations in the mouse, also affected mechanisms that regulate cellular resistance to hydrogen peroxide, cadmium, rotenone and perhaps heat and MMS. Unlike the Snell dwarf mice however, these mechanisms do not seem to affect resistance to UV light or paraquat, at least within this species set of related mammalian species.

Overall, our data are consistent with other studies demonstrating an association between mammalian life span and cellular physiology in vitro. In particular, Kapahi et al. (6) demonstrated that resistance of fibroblasts to oxidative stressors was positively correlated with mammalian longevity across several taxonomic orders. We, too, found that within the rodents (and a bat species) that resistance to hydrogen peroxide was correlated with donor maximum lifespan. Cadmium is also thought to induce cytotoxicity, in part, through the generation of reactive oxygen species (7;8). Thus, in our study it seems that an enhanced resistance to these two stressors in the long-lived species implies that the evolution of long-lived species requires augmentation of cellular pathways that regulate ROS-induced damage. However, we found that there was no relationship between life span and resistance to the toxic effects of paraquat, even though paraquat is a well-known oxidative stressor, and one to which fibroblasts from Snell dwarf mice and other long-lived mutants show in vitro resistance (8;9). This suggests there may be division among the pathways through which different oxidative stressors may mediate their action (10;11).

As discussed in Chapter I, several other groups have suggested that the repair of UV-induced DNA lesions is correlated with longevity across mammals (12-15). In our

data set, while there are clear differences between species in their resistance to UV light, across the species tested, there was no correlation between resistance to UV and species maximum longevity. This discrepancy with previous studies may be due to the parameter measured; previous studies assayed unscheduled DNA synthesis, a measure of DNA excision repair, whereas our study measures cell survival following UV irradiation. The relationship between DNA repair and cell survival is complex, particularly within the rodents; in rodent-derived cells, repair of UV lesions often does not correlate with cell survival due to relatively low levels of nucleotide excision repair in rodents (16). Further, in data sets only containing rodent species, the association between DNA repair and maximum longevity is somewhat ambiguous (17), though the number of species examined in that study is quite small. It may also be that UV resistance could have been lost with adaptation to a particular niche. Resistance to MMS, another DNA damaging agent, does tend to be correlated with longevity after most statistical analyses, suggesting that rodent longevity may require an enhanced ability to remove some types of DNA lesions. Overall, it would be informative to explore differences among these and mammalian species in the capacity of fibroblasts to repair UV or MMS-induced DNA lesions. In addition, because the data set used in this study is relatively small, it will be important to perform this study using more rodent species. It will also be of interest to analyze cellular stress resistance in other groups of closely related species, such as canines, birds, bats, or primates. All data from this study were generated using skin-derived fibroblast, a cell type unlikely to directly affect aging rate. It will therefore be important to test whether other cell types, or tissues, are similarly resistant to both lethal cellular stresses as well as agents that may have metabolic effects.

Overall, our data suggest that as rodent species evolved longer lifespan, there was also a coordinate increase in the cellular resistance to many, but not all, lethal and non-lethal agents. These data also raise many additional questions, such as whether evolution relies on these same cellular parameters to regulate longevity among other groups of animals, such as primates, or also among other groups of vertebrates such as birds. Further, the molecular processes that mediate the cellular responses to these agents still need elucidation to begin to understand their interplay with the regulation of lifespan. Thus, by studying the pathways that mediate inter-species differences in stress resistance

may help us to deconstruct this aspect of evolutionary cell biology and better understand how Nature may regulate mammalian lifespan.

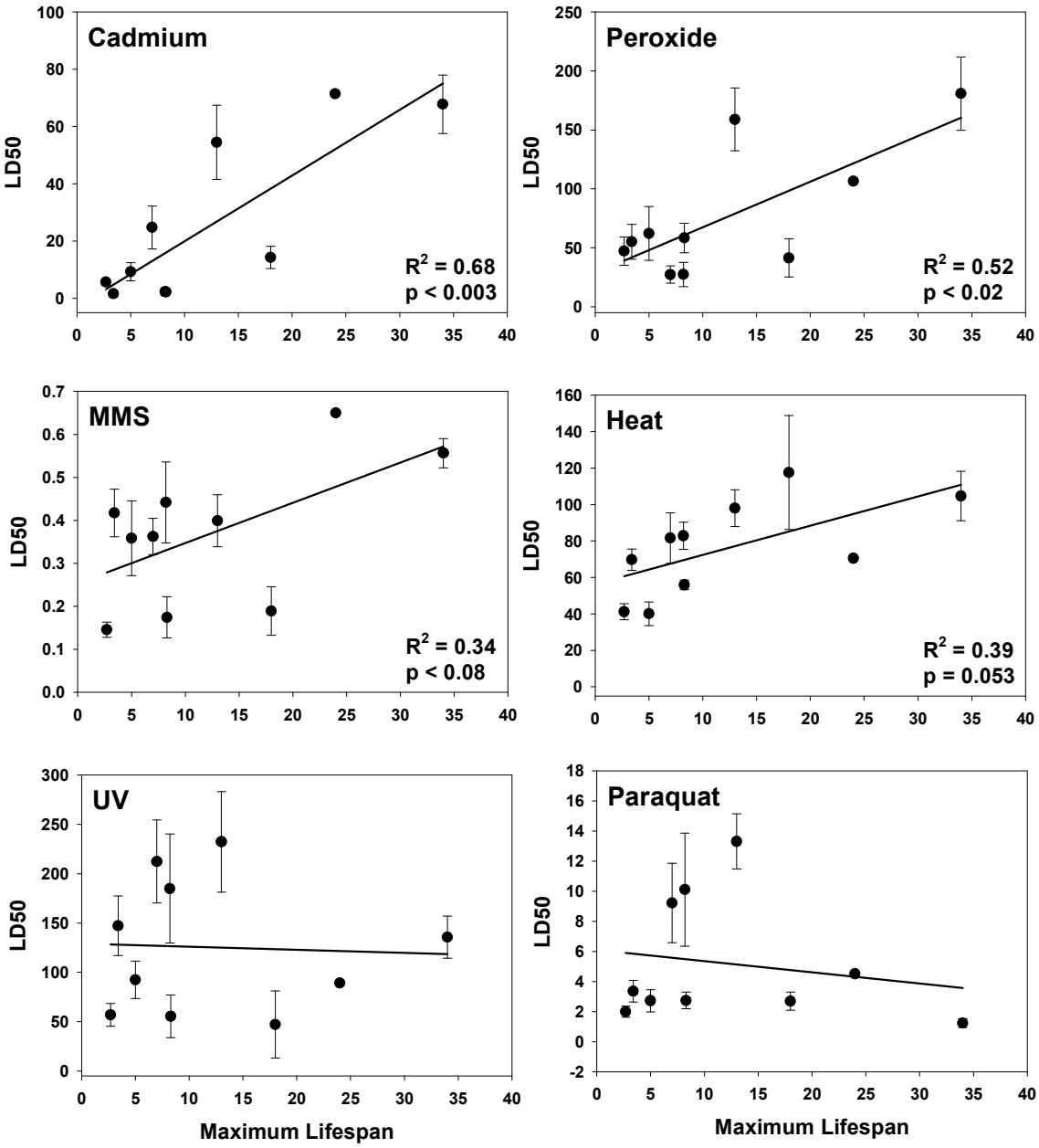

Figure 5.1. Each scatterplot shows an association between species maximum life span and mean LD50 value for each of ten species (treating laboratory mice and wild-trapped mice as separate species for reasons explained in the text). From left to right, points represent laboratory mouse, wild-trapped mouse, rat, red squirrel, white-footed mouse, deer mouse, fox squirrel, porcupine, beaver, and little brown bat. Formal species names and number of independent samples are given in Table 5.1. Error bars show standard errors of the mean. The line shows the outcome of a least squares regression. Pearson R^2 and p-values (quoted only where $p < 0.1$) reflect standard linear regression of maximum life span against mean LD50 values for the set of nine species, as in the first column of Table 5.2. Units for LD50 are in μM (cadmium and H_2O_2), mM (MMS and paraquat), J/m^2 (UV light) or minutes at 42° C (heat).

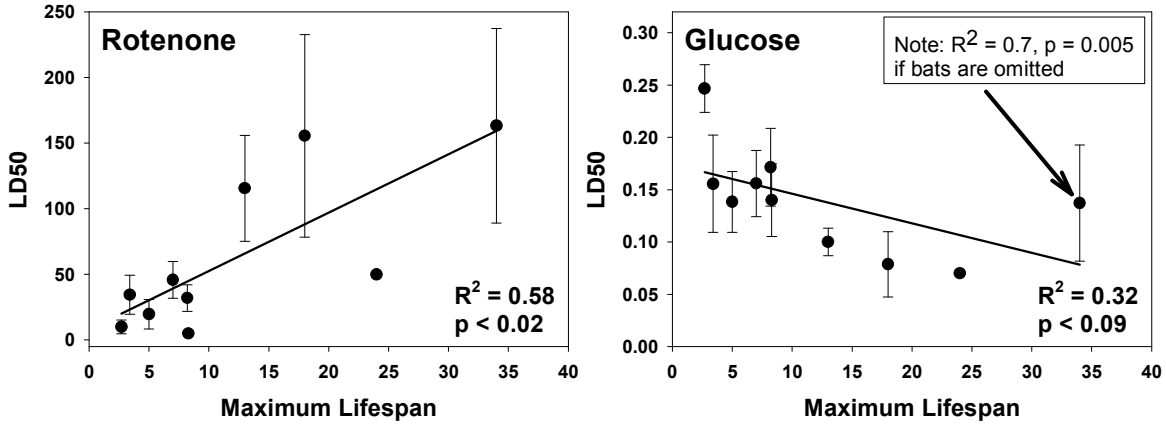

Figure 5.2. As in Figure 5.1, except that the vertical axis shows mean "ED50" values, i.e. the dose of rotenone or glucose that led to a 50% reduction in WST-1 reduction compared to cultures in control medium, for each of ten species (treating laboratory mice and wild-trapped mice as separate species for reasons explained in the text). Note that increased resistance to rotenone leads to higher ED50 levels, but that increased resistance to withdrawal of glucose is reflected by lower ED50 values, i.e. a requirement for more extreme removal of glucose to achieve equivalent metabolic inhibition. Units for ED50 are μM (for rotenone) and mg/ml (glucose).

Table 5.1: Summary of samples used in this study

Species	Common name	Life Span Estimate* (years)	Body Weight (g)	Number of samples
Mus musculus	House Mouse (lab)	2.7	41	10
Mus musculus	House Mouse (wild)	3.4	21	9
Rattus norvegicus	Norway Rat	5	200	6
Tamiasciurus hudsonicus	Red Squirrel	7	200	9
Peromyscus leucopus	White-footed Mouse	8.2	23	7
Peromyscus maniculatus	Deer Mouse	8.3	21	5
Sciurus niger	Fox Squirrel	13	800	9
Erethizon dorsatum	N. American Porcupine	18	8600	8
Castor canadensis	N. American Beaver	24	20250	1
Myotis lucifugus	Little Brown Bat	34	10	8

* With the exception of house mice, the life span estimate is the maximum recorded for each species (S. Austad, personal communication). For house mice, the life span estimate is the mean life span of the longest-lived 10% of naturally aging populations maintained in our laboratory under standard husbandry conditions.

Table 5.2: Comparison of regression methods for association between species life span and 8 tests of cell culture properties

	Simple Linear Regression	**Multi-Level Regression**	**Phylogenetically Independent Contrasts**
All Species (with lab and wild mice)			
Cadmium	**R^2 = 0.68, p = 0.003**	**p = 0.0004**	**R^2 = 0.57, p = 0.01**
H_2O_2	**R^2 = 0.52, p = 0.018**	**p = 0.015**	**R^2 = 0.43, p = 0.04**
MMS	R^2 = 0.34, p = 0.08	p = 0.18	R^2 = 0.20, p = 0.19
Heat	R^2 = 0.39, p = 0.053	**p = 0.02**	R^2 = 0.10, p = 0.37
UV	R^2 = 0.002, p = 0.90	p = 0.69	R^2 = 0.02, p = 0.66
Paraquat	R^2 = 0.03, p = 0.69	p = 0.43	R^2 = 0.07, p = 0.45
Rotenone	**R^2 = 0.58, p = 0.011**	**p = 0.045**	R^2 = 0.33, p = 0.08
Glucose	R^2 = 0.32, p = 0.087	p = 0.27	R^2 = 0.09, p = 0.41

Values in bold represent those that are statistically significant at $p < 0.05$.

Table 5.3: Regression analyses relating life span to stress resistance for wild-trapped rodents only

	Simple Linear Regression	**Multi-Level Regression**	**Phylogenetically Independent Contrasts**
Wild-Trapped Rodents			
Cadmium	**$R^2 = 0.57$, $p = 0.031$**	$p = 0.107$	**$R^2 = 0.57$, $p = 0.029$**
H_2O_2	$R^2 = 0.18$, $p = 0.30$	$p = 0.37$	$R^2 = 0.26$, $p = 0.196$
MMS	$R^2 = 0.11$, $p = 0.41$	$p = 0.29$	$R^2 = 0.37$, $p = 0.37$
Heat	$R^2 = 0.21$, $p = 0.26$	**$p < 0.0001$**	$R^2 = 0.07$, $p = 0.53$
UV	$R^2 = 0.095$, $p = 0.46$	$p = 0.53$	$R^2 = 0.09$, $p = 0.47$
Paraquat	$R^2 = 0.003$, $p = 0.97$	$p = 0.91$	$R^2 = 0.007$, $p = 0.99$
Rotenone	$R^2 = 0.29$, $p = 0.17$	**$p < 0.0001$**	$R^2 = 0.18$, $p = 0.29$
Glucose	**$R^2 = 0.81$, $p = 0.002$**	**$p = 0.0002$**	**$R^2 = 0.72$, $p = 0.008$**

Values in bold represent those that are statistically significant at $p < 0.05$.

Table 5.4: Regression analyses for residuals – body weight adjustment

	LD50 x Life Span Residual	LD50 Residual x Life Span Residual
Cadmium	**$R^2 = 0.43$, $p = 0.04$**	**$R^2 = 0.55$, $p = 0.01$**
H_2O_2	**$R^2 = 0.46$, $p = 0.031$**	**$R^2 = 0.52$, $p = 0.02$**
MMS	$R^2 = 0.16$, $p = 0.26$	$R^2 = 0.14$, $p = 0.28$
Heat	**$R^2 = 0.49$, $p = 0.025$**	**$R^2 = 0.57$, $p = 0.01$**
UV	$R^2 = 0.02$, $p = 0.69$	$R^2 = 0.03$, $p = 0.65$
Paraquat	$R^2 = 0.001$, $p = 0.93$	$R^2 = 0.01$, $p = 0.83$
Rotenone	**$R^2 = 0.56$, $p = 0.013$**	**$R^2 = 0.78$, $p = 0.001$**
Glucose	$R^2 = 0.12$, $p = 0.32$	$R^2 = 0.23$, $p = 0.16$

Values in bold represent those that are statistically significant at $p < 0.05$.

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CHAPTER VI

LONGEVITY AND STRESS RESISTANCE ACROSS MAMMALIAN SPECIES: FIBROBLASTS FROM NAKED MOLE-RATS ARE RESISTANT TO MULTIPLE FORMS OF CELL INJURY, BUT SENSITIVE TO PEROXIDE, UV LIGHT, AND ER STRESS

Abstract

The wide range of lifespan among mammalian species provides rich resources for testing ideas about the cellular and molecular properties that account for differences in aging rate. Previous work showed that skin-derived fibroblasts from mice of the long-lived mutant Snell dwarf stock are resistant, in culture, to the lethal effects of cadmium, H2O2, paraquat, UV light, heat, and the DNA alkylating agent MMS, as well as to the metabolic effects of rotenone and low-glucose media. Here we evaluated fibroblasts from young adult naked mole-rats (NMR; Heterocephalus glaber), a rodent species in which maximal longevity exceeds 28 years. Compared to mouse cells (species maximum longevity about 4 years), NMR cells were resistant to cadmium, MMS, paraquat, heat, and low glucose media, consistent with the idea that cellular resistance to multiple forms of stress may contribute to disease resistance and longevity in this species. Surprisingly, however, NMR cells were significantly more sensitive than mouse cells to H2O2, UV light, and rotenone. Furthermore, NMR cells, like cells from Snell dwarf mice, were significantly more sensitive than controls to tunicamycin and thapsigargin, which induce cell death by interfering with the function of the endoplasmic reticulum (ER stress). These data show that while longevity is typically associated with varying forms of stress resistance in mammalian models, the pattern of resistance across species may differ from that seen in single-gene mouse models of delayed aging. The heightened sensitivity of

both Snell dwarf and NMR cells to ER stress suggests that alterations in the unfolded protein response might modulate cell survival and influence the rate of organismic aging.

Introduction

The natural variation of lifespan among the species of mammals may prove to be a valuable resource for understanding the molecular mechanisms involved in regulating the aging process. Among mammals, maximum lifespan can differ by over 100 years, and even within a single order the longest-lived rodents can in some cases live more than 10-fold longer than the shortest-lived (1-3). These differences in mammalian longevity are much greater than those that can be created through single-gene mutations or dietary restriction (4). Species and mutant strains within species with exceptionally long lifespan tend to exhibit delayed rates of age-rated physical decline in multiple organ systems (3;5). Therefore, studies examining differences in the physiology and cellular biology of species that vary in their maximum lifespan may provide insights into the mechanisms that modulate the rate of age-related physiological decline.

It has long been proposed that the mechanisms that regulate the aging process may be similar to those that regulate resistance to stress (6;7). Further support for this idea has come from experimental evidence that single gene mutations that extend lifespan in the nematode *C. elegans* typically render these animals resistant to numerous forms of lethal cellular insults, including oxidative stress, UV light, heavy metals and heat (8-11). To a very limited extent, similar results have been seen in mammals; mutations in the IGF-I signaling pathway that extend mouse longevity also tend to enhance survival of mice injected with paraquat, an agent that induces oxidative stress (12-14).

In our own laboratory, we have found that fibroblasts derived from the long-lived Snell dwarf ($Pit1^{dw/dw}$), Ames dwarf ($Prop1^{dw/dw}$) and growth hormone receptor knockout (GHR-KO) mice are resistant to cell death induced by multiple agents including heat, external and internal oxidative stressors (specifically H_2O_2 and paraquat), heavy metal, UV light and the DNA alkylating agent methyl methanesulfonate (MMS) (15;16). All three of these mutant mouse lines share a phenotype of very low circulating levels of IGF-I as adults and a lifespan extension of 40-60% (17-19). Cells derived from newborn

Snell dwarf mice show little or no difference in stress resistance in comparison to cells from newborn controls, suggesting that the level of fibroblast stress resistance is regulated by post-natal factors, presumably hormonal, in these endocrine-deficient mice (16). Snell dwarf-derived fibroblasts also exhibit metabolic abnormalities elicited by exposure to non-lethal forms of stress. For example, Snell dwarf-derived fibroblasts are relatively resistant to the metabolic effects of depleting glucose from the growth media and to incubation with the mitochondrial inhibitor rotenone (20). While changes in stress resistance of skin-derived fibroblasts are unlikely to play an important causal role in the control of mouse longevity, these in vitro data suggest a model in which abnormalities of the hormonal milieu during development may lead to augmented stress resistance in a variety of cell types from long-lived mutant mice and thus contribute to the unusual delayed pathology of multiple forms of late-life illnesses in these animals (19;21;22).

Tests of stress resistance of fibroblasts derived from the skin of adult donors have also shown that maximum species longevity is correlated with cellular resistance to some forms of stress and some forms of metabolic inhibition when evaluated in multiple long- and short-lived species of rodents (23). In that study, we found that fibroblasts from long-lived species tended to be more resistant to the lethal effects of cadmium, H_2O_2, heat and MMS and also more resistant to the metabolic effects of glucose withdrawal and treatment with rotenone. Unexpectedly, we also found that there was no association between lifespan and cellular resistance to either UV light or paraquat among the species we tested. These data suggest that in vitro studies of cellular stress resistance might help to evaluate the hypothesis that shared mechanisms contribute to the regulation of longevity across the mammalian evolutionary tree.

Prompted by the results of the rodent comparison, we have now tested to see if cells from the longest-living rodents known, the naked mole-rats (NMR; (5)), are resistant to various forms of in vitro stress. The NMR (*Heterocephalus glaber)* has been observed to live to ages of at least 28 years in captivity, about seven times longer than the maximum captive lifespan of laboratory mice (5). These animals are roughly the same size as mice, yet live about ten times longer than predicted by linear regression of lifespan against size for non-volant eutherians (i.e. those that neither fly nor glide) (24;25). NMR show little physiological or reproductive decline even when approaching

30 years of age, have never been observed to develop any spontaneous neoplasm, and do not show the typical age-associated acceleration in mortality risk that characterizes nearly every other species for which detailed survival data are available (5;26;27). Thus, physiological and biochemical mechanisms have evolved in this species that can dramatically extend lifespan compared to rodents of comparable size. As such, we thought it of interest to learn if cells from NMR, like those of long-lived mutant mice, are resistant to the effects of cellular stress. Fibroblast cell lines were derived from the skin of young adult NMR to test the hypothesis that the long lifespan of these animals is accompanied by cellular resistance to stress. Our results show that cells from these animals are, as predicted, resistant to some agents, but unexpectedly, are quite sensitive to others.

Methods

Animals

NMR skin samples were obtained from a captive colony maintained in the Department of Biology at City College of New York. The husbandry conditions of these animals were as presented previously (5;26-31). Mouse skin samples were prepared from the genetically heterogeneous DC stock. DC mice are the intercrossed progeny of UM-HET3 mice, themselves generated by a cross between females of the (BALB/c x C57BL/6)F1 stock and males of the (DBA/2 x C3H/He)F1 stock. Biopsies for this study were taken from the G4 generation of DC mice. Skin samples were taken from male NMR of approximately 2 years of age and from male DC mice of 3-4 months of age. These ages were chosen as representatives of a physiologically equivalent age representing post-pubertal young adults of each species (32); 2 year old NMR have reached an age of about 7% of the species' maximal lifespan and 3 month old mice have reached an age of about 6% of the species' maximal lifespan.

Snell dwarf ($Pit^{dw/dw}$) mice, and heterozygote ($Pit^{dw/+}$) controls were bred as the progeny of (DW/J × C3H/HeJ)-*dw*/+ females and (DW/J × C3H/HeJ)F1-dw/dw males. Their sires had been previously treated with growth hormone and thyroxine to increase

body size and fertility. Tail skin biopsies were taken from male mice at approximately 3 months of age

Cell culture and establishment of cryopreserved cell lines

Tail from NMRs were sterilized with 70% ethanol wipes and biopsies of 3-5 mm in length were obtained from the distal half of the tail and placed in complete medium (CM) made of Dulbecco's modified Eagle medium (DMEM, high-glucose variant, Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma, St. Louis, MO) and 0.25 μg/ml of fungizone (Biowhittaker-Cambrex Life Sciences, Walkersville, MD) on ice and shipped overnight from New York to Michigan. For laboratory-raised DC mice and Snell dwarf and controls, tails were washed with 70% ethanol, and tail-skin biopsies of 3-5 mm in length obtained from the distal half of the tail were placed in CM prior to fibroblast isolation.

Fibroblast cultures from NMR and DC biopsies were developed using methods similar to those previously reported (15;16) except that the present study used 10% CO_2 in air and an incubator temperature of 33° C. We, like others (33), have found that fibroblasts from NMR grow poorly at the standard 37° C incubation temperature, perhaps because the animals have a body temperature several degrees below this in their natural environment (34). For this reason, both NMR and DC fibroblast cultures were incubated at 33° C. Initial cultures of cells derived from NMRs ("P0") grew to confluence at a slower rate than those of DC-derived cells (not shown) so all cells were trypsinized for subculturing when NMR cells reached approximately 90% confluence. Cell cultures were fed at day 3 (replacing 2/3rds of the medium), again at day 6 and subcultured at Day 10 at a density of 7.5×10^5 in tissue culture flasks of 75 cm^2 surface area to produce P1 cultures. This protocol was retained for all subsequent subculturing. Following an additional passage using the same protocol, P3 cells were harvested and cryopreserved as in previous studies (23). These cryopreserved cells were stored in liquid N_2 for up to 24 months before assessment of stress resistance. Cell aliquots were thawed and grown two

further passages as previously shown, with all incubations at 33° C and 10% CO_2 in air (23).

For studies of thapsigargin and tunicamycin effects, fibroblasts from Snell dwarf and littermate controls were established and cultured at 37° C as previously described (15;16).

Assessment of fibroblast resistance to lethal stress

NMR and DC cells lines were evaluated in two sets of experiments, each of which evaluated equal numbers of NMR and DC donors. P4 cultures were used to assess resistance to all agents. Tests for resistance to the lethal stresses cadmium, H_2O_2, paraquat, UV light, MMS and heat were performed as previously described (15;16;23;35). After treatment, cells were washed at 33º C with PBS, and incubated at 33º C in serum-free DMEM supplemented with 2% BSA, antibiotics and fungizone; survival was measured 18 hours later using conversion of the extracellular tetrazolium dye WST-1 to its colored formazan product (15;16;23;35). All incubations (except for heat) were at 33º C in a humidified incubator with 10% CO_2 in air.

Assessment of cell metabolism in low glucose medium and rotenone effects

For each set of glucose or rotenone assay experiments, representatives of P4 cell lines from both naked mole-rats and DC mice were assayed in parallel to minimize the effects of day to day variation. Tests for the effect of glucose withdrawal and rotenone were performed as previously shown (20;23). All incubations were at 33º C in a humidified incubator with 10% CO_2 in air. It is important to note that although WST-1 is used in tests of both lethal stresses and in the evaluation of responses to low glucose, the decline in WST-1 reduction under low glucose conditions or at the rotenone concentrations employed is not accompanied by cell death (20).

Assessment of fibroblast resistance to ER stress

For Snell dwarf and littermate control experiments, P3 cells were prepared in 96 well plates as previously described (15;16). These cells had not been previously cryopreserved and all assays with them were performed in a humidified incubator with 5% CO_2 in air and at a temperature of 37° C. Thapsigargin and tunicamycin were suspended in a solution of DMSO (Sigma) and doses of these agents were diluted in DMEM with a final DMSO concentration <0.5%. Cells were treated for 24 hours after which they were washed with PBS and incubated with serum-free DMEM supplemented with 2% BSA, antibiotics and fungizone; survival was measured 18 hours later using WST-1. DC and NMR assays for resistance to these agents were performed similarly, except all incubations and treatments were at 33º C in a humidified incubator with 10% CO_2 in air. Assays of Snell dwarf and control resistance to thapsigargin and tunicamycin were performed by Amir Sadighi Akha.

Calculation of LD50 and ED50 values, and statistical analysis

For calculation of the resistance of each cell line to chemical stressors, at each dose of chemical stressor, mean survival was calculated for duplicate wells for each cell line. The LD50, i.e. dose of stress agent that led to survival of 50% of the cells, was then calculated using probit analysis as implemented in NCSS software (NCSS, Kaysville, UT). For this analysis, extremely low doses of stress agents that caused no cell death in fibroblasts, as measured by the WST1 assay, were censored from all data sets. ED50 values for glucose withdrawal and rotenone treatments were calculated in a similar manner to estimate the level of glucose or rotenone associated with a 50% reduction in cellular metabolic activity. Because some groups of data were not normally distributed, differences between groups in mean LD50 levels were evaluated by Mann-Whitney U non-parametric tests, with each day's work containing equal numbers of cultures from each species or each genotype. For some agents, the day of assay seemed to affect the

calculated LD50 or ED50 and therefore data were also analyzed by two way ANOVA with species and day of assay as the factors.

Results

To see if fibroblasts from NMR resembled cells from other long-lived rodent species or from long-lived mutant mice (15;16;20;23), we evaluated their resistance to a series of lethal stresses and non-lethal inhibitors of cellular metabolism. Skin fibroblasts were derived in each case from young adult animals, in which approximately 5% to 10% of each species's maximal lifespan had been completed, and cell cultures for DC and NMR cultures were maintained at 33° C, a temperature at which NMR cells proliferate and survive.

The data in Figure 6.1 and Table 6.1 represent the results from cell lines tested for their resistance to the lethal agents cadmium, paraquat, heat, MMS, H_2O_2 and UV light. Our working hypothesis, based on the longer lifespan of NMR, was that fibroblasts from NMR would be relatively resistant to all these agents, similar to cells from long-lived Snell dwarf mice (15;16). Our data supported this notion for experiments assaying resistance to cadmium (Figure 6.1A), paraquat (Figure 6.1B), heat (Figure 6.1C) and MMS (Figure 6.1D) in cell lines from 11 individual animals of each species. The difference in resistance to lethal stressors ranged from 269% for paraquat to 23% for MMS. These differences reached statistical significance by non-parametric Mann-Whitney test with a $p < 0.04$ in each case.

In contrast, however, we found that NMR fibroblasts were markedly more sensitive than DC mouse fibroblasts to H_2O_2 (approximately 4-fold more sensitive, Figure 6.1E) and to irradiation with UVC light (approximately 3-fold more sensitive, Figure 6.1F). In both cases, these differences reached significance by Mann-Whitney U test with $p < 0.001$. ANOVA calculations that adjust for day-to-day variations, as compiled in Table 1, confirmed the conclusion that NMR fibroblasts were more resistant to cadmium, paraquat, heat, and MMS, and more sensitive than mouse cells to H_2O_2 and UV light, with $p < 0.01$ in each case.

The data in Figure 6.2 (and also included in Table 6.1) represent the results from cell lines tested for their resistance to the metabolic inhibition caused by withdrawal of glucose from the growth medium or by the mitochondrial inhibitor rotenone. Fibroblasts from the NMR, like Snell dwarf cells and cells from the long-lived rodents previously tested, were relatively resistant to the effect of glucose withdrawal from the growth medium (Figure 6.2A, Mann-Whitney U test $p = 0.01$). Surprisingly, however, NMR-derived fibroblasts were approximately 2-fold more sensitive than DC fibroblasts to rotenone, at the non-lethal doses used for these tests (Figure 6.2B, Mann-Whitney U test $p = 0.003$). An ANOVA approach confirmed both inferences (Table 6.1).

The LD50 and ED50 values calculated for the resistance of mouse cells to most agents were similar to those noted in previous studies which had used cultures grown at 37° C, except for somewhat increased resistance to cadmium and rotenone (15;16;20;23;35). To see if these two disparities might be due to the lower growth temperature used in the current set of experiments, we evaluated stress resistance in a set of cell lines from DC mice assayed in parallel under growth conditions of 33° or 37° C, with 10% CO_2 in air. The 4° temperature decrease alone resulted in a mean LD50 and ED50 of approximately 10-fold higher for cadmium and rotenone resistance, and 2-fold increase for resistance to peroxide; there was no difference in resistance to UV light (data not shown).

It seemed plausible that some of the agents used in this and our other studies (15;16;20;23) might act in part by inducing ER stress (36-38), a term coined to signify an imbalance between cellular demand for ER function and ER capacity (39). As a prelude to assessing NMR cells for resistance to ER stress agents, we compared the lethal effects of thapsigargin and tunicamycin on fibroblasts from Snell dwarf mice and their littermate controls. Thapsigargin disrupts ER Ca^{2+} homeostasis through irreversible inhibition of the endoplasmic reticulum Ca^{2+}-ATPase (40), which in turn interferes with the function of Ca^{2+}-sensitive chaperones such as calreticulin and ERp57, and hampers the post-translational processing, folding and export of proteins from the ER (41). Tunicamycin abolishes N-linked glycosylation of proteins in eukaryotic cells by blocking the function of N-acetylglucosamine-1-transferase, and thus interferes with the assembly and transport of glycoproteins from the ER to the Golgi complex (42;43). Figure 6.3 shows the results

of six experiments involving cell lines derived from 8 or 9 pairs of Snell dwarf and control mice, showing that, in contrast to their relative resistance to most sources of lethal injury, Snell fibroblasts are significantly more sensitive than cells from littermate controls to cell death induced by thapsigargin (Figure 6.3A, Mann-Whitney U test $p = 0.002$) or tunicamycin (Figure 6.3B, Mann-Whitney U test $p = 0.03$). To see if NMR cells were, like Snell dwarf fibroblasts, unusually sensitive to agents that induced death via ER stress, we evaluated six pairs of NMR and mouse DC cultures. Figure 6.4 shows that NMR cells were indeed significantly more sensitive to both thapsigargin (Figure 6.4A, Mann-Whitney U test $p = 0.01$) and tunicamycin (Figure 6.4B, Mann-Whitney U test $p = 0.004$) compared to cells from DC mice tested in parallel. These data are also included in Table 6.1.

Discussion

In this study, we tested the hypothesis that fibroblasts derived from the skin of the longest-lived rodent, the NMR, exhibit enhanced resistance to lethal and non-lethal cellular stresses as has been reported for cell lines from long-lived mutant mice (15;16;20) and for cell lines from long-lived rodent species (23). We found that NMR-derived cells were indeed significantly more resistant than mouse cells to some forms of stress, i.e. cadmium, paraquat, heat, and MMS, and relatively resistant to the non-lethal metabolic effects of low glucose concentrations. Surprisingly, however, we found that cells from NMRs were significantly more susceptible than mouse cells to the effects of H_2O_2, UV light and rotenone. This unexpected pattern of differential resistance raises a number of questions about the extent to which regulated responses to various forms of stress are coordinated, and provides some insights into the potential role of stress resistance in the evolution of NMR longevity.

Interspecies comparisons can sometimes be confounded by differences in body size and the multitude of parameters that scale with size, such as metabolic rate. Larger species of mammals tend to live longer, so that almost any trait that is linked to body size is likely to show a correlation with lifespan (25;44). Interpretation of NMR results, however, is not confounded in this manner, because adult NMR (~35 g) are similar to

mice in size (~40 g for adult DC mice) (5;45). Our design also focused on comparisons between cells taken from mice and NMRs that were at equivalent stages of development and at physiologically equivalent ages, i.e. from young adult animals that had reached full maturity and had lived ~6% of their lifespan. At these ages (3-4 months for mice and ~2 years for NMRs), neither mice nor NMR have yet begun to show any of the signs of aging, including increased mortality risk (5).

Our previous work has shown that fibroblast resistance to the lethal effects of cadmium, heat, and MMS toxicity and to the metabolic effects of glucose withdrawal from growth medium show significant or suggestive correlations with maximum longevity of the donor species (23). Fibroblasts from NMR are relatively resistant to each of these agents and are also resistant to paraquat. These data suggest that there may be pathways that regulate the resistance to these agents in parallel, and that the level of activity could vary with maximum lifespan among rodent species. These same hypothetical pathways may be those that produce resistance to these forms of stress in cells from Snell and Ames dwarf mice and from GHR-KO mice (15;16;20). Studies of cell lines from normal genetically heterogeneous mice have shown that those individual mice whose skin-derived fibroblasts are resistant to cadmium are also resistant to low glucose, again consistent with the idea that resistance to these two forms of stress is co-regulated, perhaps involving effects of polymorphic loci (20).

Many experts have proposed that cellular damage due to oxidative stress contributes to many of the diseases of aging, and that resistance to oxidative damage is a key element in extended longevity within or across species (46;47). Studies similar to those presented here have shown that primary fibroblasts from long-lived animals tend to be resistant to peroxide damage in comparisons of mammals across many taxonomic orders (48), within the rodents alone (23) and in long-lived mutant mouse strains (15;16). Some tissues from the NMR also seem to be resistant to oxidative injury, including arterial endothelial tissue and mitochondrial membranes from the liver and muscle (27;31). Our results showing that fibroblasts from NMR are resistant to cell death induced by paraquat are consistent with this model (Figure 6.1B). It is thought that paraquat causes acute cellular toxicity through the production of superoxide radicals in

the mitochondria, which in turn result in disruption of NADPH-requiring biochemical processes (49;50).

In contrast, however, NMR cells were dramatically more sensitive than mouse cells to the lethal effects of H_2O_2, and are indeed more sensitive to this agent than any other rodent we have previously studied (23). This result is provocative because these results do not support most models of oxidative stress and longevity. Explanations for this disparity may lie in the particulars of both species-specific oxidative damage and the conditions under which our assays are performed. The toxicity of H_2O_2 differs from that of paraquat in that H_2O_2 causes oxidative stress through the production of hydroxyl radicals that damage the integrity of membranes, lipids and nucleic acids (51), damage that may be exacerbated by the serum-free conditions of our assay (52;53). Recent studies have shown higher levels of oxidative damage to proteins and nucleic acids and particularly to lipids in tissues from NMR compared to mice of the same chronological or physiological age (29;30). If NMR-derived fibroblasts exhibit a similar phenotype, these cells may be sensitive to the effects of further oxidative damage, such as that caused by H_2O_2. In theory, this sensitivity could be distinct from the actions of paraquat and other oxidative stressors upon NADPH-dependent processes.

NMRs exhibit many characteristics that are contrary to those predicted by the oxidative stress theory of aging, in addition to the relatively high levels of oxidative molecular damage just mentioned. For example, mice and NMR show similar levels of ROS production in heart mitochondria (AJ Lambert, R. Buffenstein, unpublished) despite their disparities in maximum longevity. Similarly, NMRs have activity levels in the liver of the antioxidants superoxide dismutase, catalase, and glutathione that are similar to, i.e. not higher than, those seen in mice (28;30). In addition, glutathione peroxidase activity is much lower in NMR livers compared to that observed in livers from mice (28). Peroxide treatment in vitro induces lipid peroxidation and DNA oxidation that cause cell death (54), effects that can be modulated in part by the activity of glutathione peroxidase (55). It is not known if NMR fibroblasts have lower levels of superoxide dismutase, catalase, and glutathione peroxidase than do mouse cells, but such differences might in principle contribute to the sensitivity of these cells to peroxide exposure in vitro. It seems clear from the NMR fibroblast data that resistance to H_2O_2 and resistance to paraquat are likely

to be regulated by different defense mechanisms. Our data support the general conclusion that the exceptional lifespan of NMR is not likely to reflect merely a dramatic resistance to all forms of oxidative damage (28-30).

Loss of cellular resistance to H_2O_2 and UV light may reflect evolutionary adaptations to the subterranean niche of the NMR in its natural environment, where the gaseous atmosphere within the burrows is both hypoxic and hypercapnic (56). Fossil records reveal that Bathyergid ancestors have inhabited a subterranean milieu since the early Miocene (ca 24 million years ago; (57)). These animals have evolved a set of morphological and physiological phenotypes well suited to life underground, such as a markedly atrophied visual cortex and expanded somatosensory cortex, in keeping with sensory needs in the dark, and their skin is poorly pigmented (58). Physiological adaptation has included low rates of gas exchange and heat production leading to low body temperatures, as well as tolerance of hypoxia and hypercapnia (59;60). Adaptation to a hypoxic environment also may have driven the loss of cellular defenses to peroxide discussed above. Similarly, sensitivity to UV light may result from evolving in an underground environment in which sunlight-induced damage rarely occurs. The repair of UV genomic lesions is closely correlated with survival of cells following irradiation and occurs primarily through nucleotide excision repair, the rate of which is thought to be limited by the recognition of genomic lesions (61;62). Nucleotide excision repair also recognizes lesions caused by agents other than UV light, and it has been shown that cells that have lost the ability to repair UV-induced damage nevertheless can still repair these other lesions with varying levels of efficiency (63). It would be of interest to test if skin-derived NMR cells, which we show here are sensitive to UV light, are able to resist the toxic effects of other DNA lesions repaired by NER such as the interstrand cross-links induced by cisplatin. It would also be of interest to test if cells derived from NMR internal organs display sensitivity to UV light compared to those from other rodents. Alternatively, it might be possible that the UV sensitivity of NMR-derived cells could contribute to the remarkably low rate of spontaneous cancer in these animals (5); enhanced apoptosis of cells with damaged genomes might inhibit the development and progression of neoplasia (64).

NMR-specific cellular responses to some of these agents may result from altered cellular metabolism. Cells from poikilothermic reptiles tend to exhibit small inner mitochondrial surface area and greater membrane permeability of the inner mitochondria compared to mammals, and both traits are associated with decreased mitochondrial activity (65;66). Because NMRs are thermally labile mammals outside the warm confines of their thermally buffered equatorial burrows, they may exhibit mitochondrial phenotypes similar to those seen in other poikilotherms in other classes of vertebrates, and this may result in decreased metabolic activity and metabolite levels. The non-lethal effects measured by the rotenone assay may be regulated by cues from the mitochondria, including the levels of metabolic products such as NADH and NADPH (20), and thus related to metabolic activity. Diminished activity of mitochondria may also contribute to resistance to the toxicity of paraquat; the mechanism of paraquat-mediated cell death is most often attributed to its induction of superoxide formation in the mitochondria (49). Investigation into the nature of mitochondria in these animals may prove useful in understanding the relationship between the resistance (or sensitivity) to these agents and longevity.

Phylogenetic history may also contribute to the unexpected sensitivity of NMR cells to H_2O_2 and UV light. NMRs are members of the rodent suborder Hystricognathi and are more closely related to other rock rats, cane rats, guinea pigs and porcupines than to mice, rats and other rodents in other suborders (5). Our previous comparison of cells from multiple rodent species included only a single species of hystricognath, the North American porcupine (23). Although the data set as a whole showed a correlation between peroxide resistance and longevity, the average resistance of cells from the long-lived porcupine (Mean ± SE, 41 μM ± 15) was not different from those from laboratory-derived DC mice (47 μM ± 12, t-test $p = 0.8$) or from cells derived from wild-caught mice (55 μM ± 14, t-test $p = 0.5$), both of which are much shorter-lived than porcupines. Further, cells from porcupines (47 J/m^2 ± 34) tended to be sensitive to UV light compared to cells from DC mice (57 J/m^2 ± 12, t-test $p = 0.8$) and were significantly more sensitive than cells from wild-caught mice (147 J/m^2 ± 28, t-test $p = 0.04$). Glutathione peroxidase activity has been shown to be low in tissues from the hystricognath guinea pig and NMR compared to mice and rats (28;67-70) which might contribute to a relative sensitivity to

peroxide among the members of the suborder. It is plausible that these two suborders may have diverged, early in their history, in the biochemical factors by which they respond to various forms of cell injury. It would thus be of interest to evaluate stress resistance of cells derived from hystricognath species whose lifespan was shorter than NMR and porcupines.

As part of this study, the compounds thapsigargin and tunicamycin were used to examine the effect of ER stress on fibroblasts from dwarf mice and NMR. Both of these agents act to hamper the transport of proteins from the ER, thapsigargin by disrupting ER Ca^{2+} homeostasis and tunicamycin through abolishing N-linked glycosylation of proteins (71). To our surprise, fibroblasts from both dwarf mice and NMR proved to be more sensitive to the induction of ER stress than their respective controls. The accumulation of unfolded or misfolded proteins in the endoplasmic reticulum causes ER stress, and instigates a concerted adaptive program known as the unfolded protein response (UPR). The UPR alleviates ER stress by down-regulating the synthesis of secreted proteins, up-regulating ER chaperone and foldase expression levels, and activating ER-associated degradation (ERAD), thereby easing the burden on the stressed ER by reducing its protein load, increasing its folding capacity, and eliminating irreparably misfolded proteins (39;72;73). In higher eukaryotes, PERK, a double-stranded RNA-activated protein kinase-like ER kinase, is the proximal transducer of protein synthesis inhibition, whereas ATF6, a basic leucine zipper transcription factor, and IRE1, a protein kinase/endoribonuclease, orchestrate the up-regulation of chaperones and foldases, and activation of ERAD. If these pro-survival efforts are overwhelmed, ER stress-related apoptosis ensues.

Recent studies tend to implicate ER stress in the pathogenesis of many late-life illnesses (39;74). It has also been contended that the ER stress response might affect the aging process itself (75). So far, the *C. elegans* model provides the most compelling evidence in this respect (76). Treatment of *C. elegans* with the small molecule resveratrol extends life span in a *daf-16*-independent manner. Resveratrol induces the transcription of *abu-11* (activated in blocked UPR), a member of a gene family with a role in the ER stress response. RNA interference data have shown that the resveratrol effect requires *abu-11*, and that worms overexpressing *abu-11* displayed long lifespan.

Furthermore, age has been shown to modulate certain effectors of the UPR. For example, age leads to a decline with age in eIF2α phosphorylation together with an increase in GADD34 levels (77), as well as an increase in CHOP and phospho-JNK levels (77;78) in rat livers. Aging in mice also leads to carbonylation of BiP, protein disulfide isomerase (PDI), and calreticulin in the liver (79). Our data show that dwarf and NMR fibroblasts are unusually sensitive to the lethal effects of ER stress. It is possible that this sensitivity, if it occurred in appropriate cells in vivo, might help to rid the organism of defective cells and thus postpone pathological outcomes. It is also possible that heightened sensitivity to ER stress might induce compensatory increases in pathways that protect against the lethal effects of some of the other agents tested in our in vitro analyses. More information on the molecular basis for sensitivity of dwarf and NMR cells to thapsigargin and tunicamycin, and on the relationship of the UPR to cellular responses to other forms of stress, is thus likely to be informative.

Understanding what mechanisms Nature uses to create long-lived species is a fundamental problem in biogerontology. Overall, the data presented here contribute to the growing evidence associating unusual longevity with resistance to cellular injury, both within and across species. Disparities among multiple models shown of long life, however, suggest that the specific pattern of cellular resistance may well vary from clade to clade. Developing methods to extend these analyses to other cell types, *in vitro* and in intact animals, is likely to provide additional insight into these relationships. Our data further illustrate the value of using non-traditional models, such as the NMR, for tests of specific hypotheses about the control of aging.

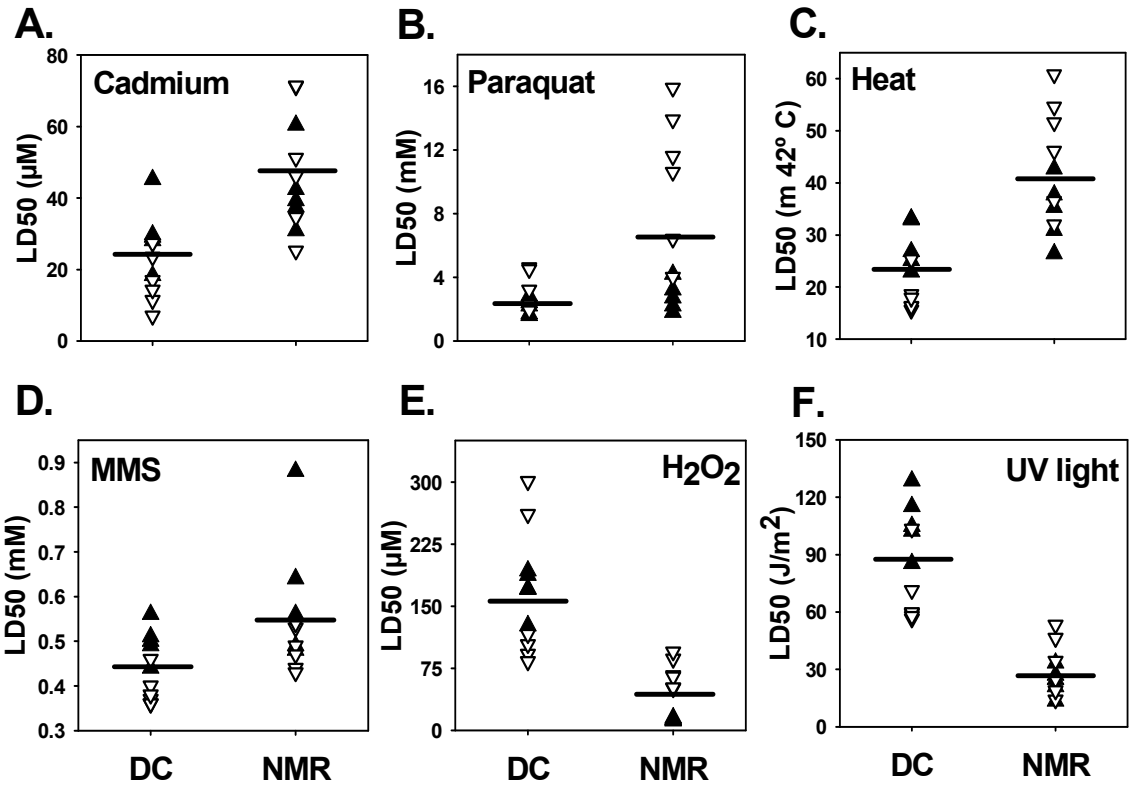

Figure 6.1. Skin-derived fibroblasts from naked mole-rats are resistant to many forms of lethal stress, but sensitive to H_2O_2 and UV light. Each symbol in the figure represents fibroblasts isolated from an individual NMR or laboratory mouse (DC); the horizontal line indicates the mean value for each group. All data values were collected from assays performed on two separate days; the day of assay is represented in these figures by open or closed triangles. The p-values for the Mann-Whitney U statistic are shown in Table 6.1; N = 11 for each group.

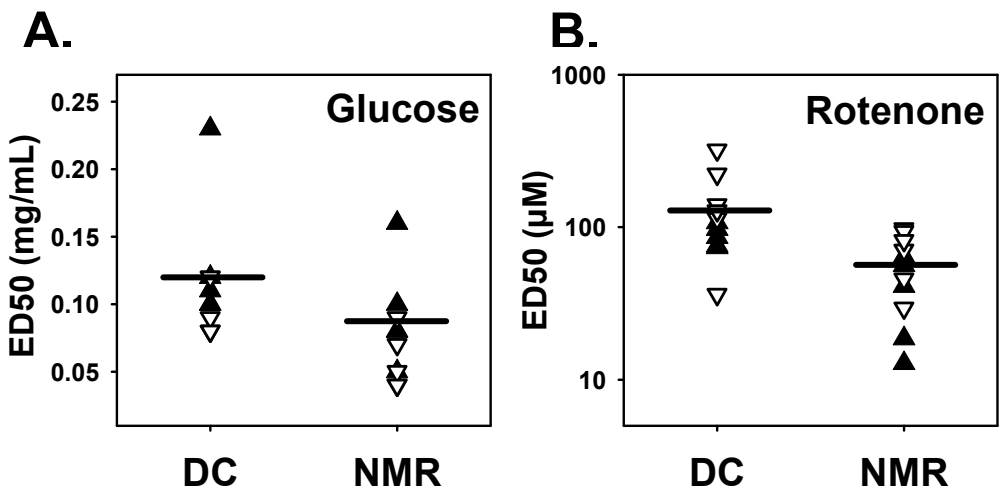

Figure 6.2. Skin-derived fibroblasts from naked mole-rats are resistant to glucose withdrawal from growth media, but sensitive to rotenone. Each symbol in the figure represents fibroblasts isolated from an individual NMR or laboratory mouse (DC); the horizontal line indicates the mean value for each group. All data values were collected from assays performed on two separate days; the day of assay is represented in these figures by open or closed triangles. The p-values for the Mann-Whitney U statistic are shown in Table 6.1. N = 10 for glucose; N = 11 for rotenone.

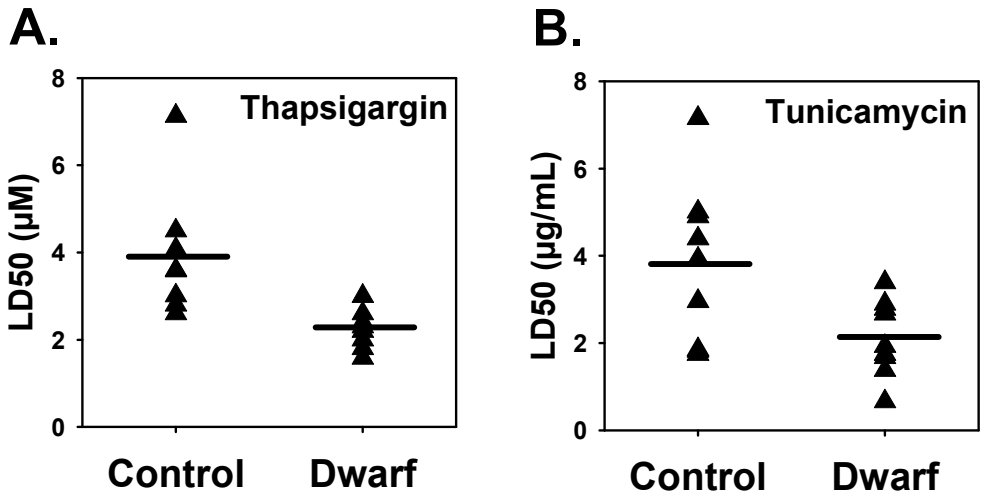

Figure 6.3. Skin-derived fibroblasts from long-lived Snell dwarf mice are sensitive to the lethal effects of agents that induce ER stress. Each symbol in the figure represents a cell line isolated from a separate Snell dwarf (Dwarf) or littermate control (Control) assayed for resistance to thapsigargin (N = 8) or tunicamycin (N = 9). The p-values for t-tests comparing dwarf to normal were $p = 0.002$ for thapsigargin and $p = 0.03$ for tunicamycin. The horizontal lines represent mean values for each group. These data were generated by Amir Sadighi Akha in our laboratory.

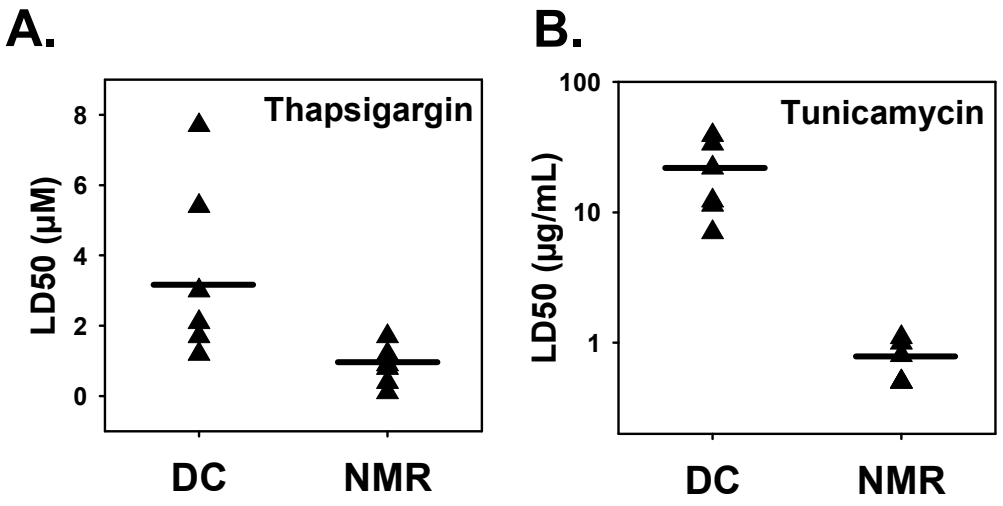

Figure 6.4. Skin-derived fibroblasts from naked mole-rats are sensitive to the lethal effects of agents that induce ER stress. Each symbol in the figure represents a cell line isolated from a separate NMR or laboratory mouse (DC) that was assayed for resistance to thapsigargin or tunicamycin. The horizontal lines represent the mean value for each group. The p-values for the Mann-Whitney U statistic are shown in Table 6.1; N = 6 for each group.

Table 6.1. Resistance to lethal and non-lethal agents of skin-derived fibroblasts from the naked mole-rat and laboratory mice

Treatment	Mean LD50 or ED50 (SE) NMR	Mean LD50 or ED50 (SE) DC	Sample Size	Mann-Whitney U p value	Two Way ANOVA F (p) Species	LD50 ratio NMR:DC
Cadmium (μM)	46.4 (2.7)	22.8 (3.1)	11	<0.001	17.1 (<0.001)	2.03:1
Paraquat (mM)	7.0 (0.6)	2.6 (0.5)	11	0.02	13.9 (<0.001)	2.69:1
Heat (m 42°)	41.3 (1.6)	22.8 (1.5)	11	<0.001	33.1 (<0.001)	1.81:1
MMS (μM)	540.9 (6.7)	439.1 (16.7)	11	0.04	7.8 (0.01)	1.23:1
Peroxide (μM)	43.8 (3.0)	164.2 (39)	11	<0.001	30.0 (<0.001)	**0.26:1**
UV light (J/m^2)	28.3 (2.6)	84.8 (7.4)	11	<0.001	85.1 (<0.001)	**0.34:1**
Glucose (μg/mL)	76.0 (2.4)	115.0 (8.4)	10	0.01	5.4 (0.03)	1.50:1*
Rotenone (μM)	55.6 (4.9)	127.3 (40.2)	11	0.003	8.7 (0.009)	**0.44:1**
Thapsigargin (μM)	0.85 (0.11)	3.5 (1.0)	6	0.01	NA	**0.26:1**
Tunicamycin (μg/mL)	0.73 (0.05)	20.9 (5.3)	6	0.004	NA	**0.03:1**

The Mann-Whitney U column presents the p-value for a two-tailed Mann-Whitney U test between species. The "Two Way ANOVA" column presents the F statistic from an analysis of variance using donor species and day of experiment as the factors; the p-value corresponds to significance for the "species" term. The ANOVA approach was not used for the thapsigargin and tunicamycin experiments (NA,not applicable) because all assays were performed on the same day for these agents. Ratios in boldface are those for which NMR cells were more sensitive than mouse cells. For glucose withdrawal, a lower ED50 represents greater resistance to this treatment (*).

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CHAPTER VII

CONCLUSIONS

Key findings

The intent of this project was to explore molecular mechanisms that may help us learn more about the regulation of the aging process. Prompted by reports from invertebrates, we wished to explore if mutations that extend lifespan in mice also confer cellular resistance to stress. The experiments presented in the preceding chapters were thus designed to address three primary aims: to test whether cells from long-lived genetic mutant mice are resistant to stress, to test whether cells from long-lived mutant mice exhibit differences in the repair of DNA damage relative to those from control mice, and to test if the evolution of long-lifespan across mammalian species is correlated with cellular resistance to stress.

The main finding of this thesis is that the mutations that extend lifespan in mice also render cells from those mice resistant to multiple forms of cellular stress. In our initial report from the lab presented in Chapter II, we demonstrated that fibroblasts derived from the skin of young adult Snell dwarf mice and grown in culture through multiple passages, are resistant, in vitro, to cell death induced by multiple cytotoxic agents including hydrogen peroxide, paraquat, cadmium, heat and UV light (1). In this study, we documented stress resistance of cells from Snell dwarf mice on two separate genetic backgrounds, suggesting that phenotype was specific to the Snell dwarf mutation. These findings suggested that cellular characteristics of long-lived animals might be retained by cells in culture and thus, stimulated our interest in exploring if cellular stress resistance was a common feature of other long-lived genetic mutant mice. We then demonstrated that cells from long-lived Ames dwarf mice were similar to those from Snell dwarfs in that they were resistant to the lethal effects of hydrogen peroxide,

cadmium, UV light and paraquat; resistance to heat was not tested (2). These results confirmed our earlier findings in the Snell dwarf and also suggested that the association of cellular resistance to mouse lifespan is replicable for separate longevity-extending mutations on mice with different genetic backgrounds and breeding schemes. Because Ames dwarf and Snell dwarf mice have different mutations within a shared pathway critical to development of the embryonic pituitary gland, these mice share an altered hormonal profile that features declines in circulating levels of growth hormone (GH), thyroid stimulating hormone and prolactin. Secondary effectors of these hormones are also diminished, including insulin-like growth factor I (IGF-I). Because of these shared phenotypes, it was not unexpected that both the Ames and Snell dwarf mice displayed similar patterns of cellular stress resistance. In the same report we also found that fibroblasts from GH receptor knockout (GHR-KO) mice were resistant to hydrogen peroxide, paraquat and UV light but not resistant to cadmium toxicity (2). GHR-KO mice differ in their hormonal profile from Ames and Snell dwarf mice, in that GHR-KO mice have high levels of GH and only modest declines in thyroid hormones, but like Ames and Snell dwarf, GHR-KO mice have very low levels of circulating IGF-I (3-5). Thus, these observations confirm that cellular resistance to stress is enhanced in long-lived genetic mutations other than the hypopituitary dwarf models. Further, the observation that cells from Ames dwarf, Snell dwarf and GHR-KO are all resistant to multiple forms of stress suggests that early-life IGF-I deficits in the animal may underlie the regulation of these cellular characteristics. These results are consistent with the findings in *C. elegans* that deficiencies in the insulin/IGF-I-like signaling pathways can extend worm longevity and render worms resistant to multiple types of stress (6-11). Because deficiencies in this pathway share phenotypes across multiple phyla, these results suggest that the regulatory effects of insulin/IGF-I-like signaling on lifespan and stress resistance have been established since the early evolution of eukaryotes.

Our observations also suggest that the mechanisms of longevity exert effects on multiple pathways of stress resistance. Some have predicted that the accumulation of specific forms of damage, such as oxidation or DNA damage and mutations, are the most important regulators of aging (discussed in Chapter I). Our results suggest that a better indication of lifespan may be a coordinate upregulation of resistance to multiple forms of

cellular damage. Cells from Snell dwarf mice are resistant both to oxidative stressors, like hydrogen peroxide and paraquat, and to agents that may act at least partially independent of oxidative stress, such as cadmium, heat and UV light. In Chapter III, we found a positive correlation between resistance to peroxide and resistance to paraquat, cadmium and UV light (12). These results suggest that there may be overlapping sets of mechanisms that control these cellular properties. In Chapter II, we demonstrated that antioxidants protected fibroblasts from peroxide, paraquat and to some extent cadmium, but provided no protection from UV-induced death (2). Further, we showed that Snell dwarf-derived cells were resistant to the lethal effects of the DNA damaging agent methyl methanesulfonate (MMS) which is thought to act independently of oxidative stress (2). We selected a range of lethal agents to test, as shown in Fig. 7.1, because each of these agents leads to cell death through distinct, though in many cases partly overlapping, molecular mechanisms. Thus, we infer that the mechanisms that extend longevity in the Snell dwarf affect multiple pathways of cellular stress resistance in parallel, including those that induce cell death through oxidation and those, like UV light and MMS, that act independently of oxidative stress. Therefore, these results suggest it is unlikely that the accumulation of any single form of macromolecular damage is the key regulator of aging, but rather that lifespan extension may be accomplished by protection from multiple types of damaging lesions. To some extent, biochemical data from the Ames dwarf mice support this theory; others have shown that a number of tissues from dwarf animals have higher expression of not only antioxidants, but also heat shock proteins and metallothionein (13-16).

The mechanisms by which resistance to multiple forms of stress are cooridinated are unclear, but one possibility might be the through activation of transcriptional factors that act on many genes in parallel. Single gene mutations in the C. elegans IGF/insulin-like signaling pathway of the pathways can extend longevity of the adult worm and increase resistance to multiple forms of stress including heat, oxidative stress, UV irradiation and heavy metals (6;10;11;17-19). The transcription factor daf-16 is negatively regulated by the insulin pathway and mutations in this gene suppress the increase of longevity and stress resistance of some long-lived worm IGF/insulin-like signaling mutants (10;11;17-20). In addition, overexpression of this transcription factor

alone can increase lifespan and stress resistance (21). Several genes encoding proteins thought to protect the cell from cellular stress have been shown to be downstream of daf-16, including superoxide dismutase, metallothionein, and the heat shock proteins HSP-16, HSP-70 and HSP-90 (10;18;22-24). The activity of the mammalian orthologue of daf-16, the transcription factor FOXO3A, has also been shown to regulate DNA repair processes and resistance to oxidative stress (25-29) and has also been recently shown to regulate the process of autophagy, a primary defense response to certain forms of cellular stress (30). Like daf-16, the activity of FOXO3A is regulated by signal transduction through the IGF/insulin signaling pathway (31). Thus, it may be that diminished IGF-I during the early life development of Snell dwarf mice may alter the cellular localization of FOXO3A, and thereby alter the expression of genes that regulate longevity and those that might control cellular resistance to stress (Figure 7.2). In C. elegans, the lifespan extension and stress resistance conferred by the activity of daf-16 can also be regulated by inputs that are independent of IGF/insulin signaling. For instance, overexpression of the sirtuin sir-2 extends lifespan and increases stress resistance in a daf-16 dependent manner (32;33). The mammalian orthologue of sir-2, SIRT1, has also been shown to regulate cellular stress resistance through FOXO3A (27;28). In addition, overexpression of JNK can extend worm and Drosophila lifespan and increase thermotolerance; thermotolerance of JNK overexpressing worms seems to require daf-16 activity and lifespan extension of JNK overexpressing flies requires Drosophila FOXO (34-36). However, some have suggested that JNK may in part exert its lifespan extending effects by antagonizing signals through the IGF/insulin signaling pathway (36). Signaling through the IGF/insulin signaling pathway may also act independently of daf-16/FOXO. TOR kinases respond to nutrient signals in part mediated by insulin-like signaling, and in C. elegans deletion of TOR kinase activity can extend lifespan and confer heat resistance (37-39). In Ames dwarf mice, there is a downregulation of the translation regulatory signaling pathways of PI3K, Akt, and mTOR suggesting a possible mechanism for extended longevity and cellular stress resistance of this particular long-lived mutant mouse (2;40). Thus, it would be of interest to determine if Snell dwarf mice, or if cells derived from Snell dwarf mice, differed in their regulation of JNK, mTOR, SIRT1, and particularly FOXO3A. Conversely, it would be of interest to determine if altering the

activity of these proteins, either genetically or pharmacologically, might increase stress resistance in cell culture or extend lifespan in mice,

Our data on dwarf and GHR-KO cellular stress resistance also suggest that the long life span and exceptional disease resistance of these mice may be due to cellular resistance to stress in vivo. Others have shown that dwarf mice, but not GHR-KO mice, are resistant to death from paraquat injection (41;42). For reasons discussed in Chapter I, survival after paraquat, or other oxidative stressors, is unlikely to be an accurate measure of stress resistance of an animal. However, the delayed progression of some age-related pathologies give hints that dwarf mouse cells are stress resistant in vivo. For instance, old dwarf mice have significantly fewer cataracts than control mice (43) and cataracts have been shown to be caused by the accumulation of oxidative damage (44), suggesting that the lens of dwarf mice may be resistant, in vivo, to oxidative stress relative to those in control mice. Similarly, the development of many forms of neoplasia might reflect the accumulation of DNA damage and resulting genome instability (45;46). Both dwarf models have diminished incidences of spontaneous cancer (43;47), and Snell dwarf mice develop fewer tumors following treatment with chemical mutagens (48-50). While we cannot be certain these are caused by differences in DNA repair or stress resistance in vivo, delayed spontaneous and induced cancer rates hint at fundamental differences in the maintenance of the genome between long-lived and control mice. For future work on stress resistance in dwarf mice, it would thus be of interest to develop assays of in vivo stress resistance so that some of these questions may be addressed. This is discussed in greater detail below.

As part of this thesis, we also found that the Snell dwarf fibroblasts are more proficient than control cells at removing UV lesions from their nuclear DNA, thus providing a possible mechanism for their resistance to UV light. As discussed in Chapter IV, we showed that Snell dwarf fibroblasts had a significantly faster recovery from UV-induced transcription inhibition. We also observed that the removal of UV lesions from the genome was greater in cells from Snell dwarf-derived fibroblasts than from control cells. Cell death caused by UV light is, at least in part, due to the cytotoxicity of bulky UV-induced lesions formed within actively transcribing genes; these adducts block the progression of RNA polymerase and inhibit transcription (51;52). Cells from donors

with defects in transcription coupled repair have been shown to have poor recovery of transcription following UV and undergo apoptosis at relatively low doses of UV radiation (52;53). Thus, it may be that relative UV resistance of dwarf-derived fibroblasts is due to an enhancement of the transcription coupled repair process. We also showed that the increased DNA repair in dwarf-derived cells may be due to greater expression of two nucleotide excision repair proteins, XPC and CSA, when cells are deprived of serum.

It is not entirely clear how lifespan may be extended by such enhanced repair. The effects of transcription coupled repair have largely been observed in response to UV light (54); beyond the development of skin cancers, UV-induced damage seems unlikely to play a large role in regulating the rate of aging. However, transcription coupled repair has been shown to repair oxidation and alkylation lesions (55-57) suggesting that alteration of repair rates could affect cellular resistance to other forms of stress and in other cell types that are not exposed to UV irradiation. In relation to these reports, we also found (but did not present in Chapter IV) that Snell dwarf-derived fibroblasts recover from MMS-induced transcription inhibition more rapidly than control. It may be that enhanced DNA repair of Snell dwarf mice, in vivo, protects their cells from apoptosis due to the accumulation of genomic damage, and thus the function of tissues in which cell replacement is limiting is preserved. We also showed that dwarf-derived cells were able to remove UV lesions from their genome by global genome repair relatively more rapidly than control-derived cells. Lesions in the 95% of the genome that is not transcribed in a given cell type are considered primarily mutagenic and thus the enhanced removal of these lesions might contribute to the low cancer incidence of dwarf mice (43;54;58). It may that enhancement of both types of repair in dwarf mice may reduce both the age-related increase in tissue dysfunction and the incidence of cancer which together might contribute to the longevity of these mice. It may then be important in future studies to determine if there are differences in DNA repair between Snell dwarf mice and control in vivo. To address this, it would be of interest to measure the formation and kinetics of repair of UV lesions in the skin of irradiated mice (discussed below). Alternatively, the repair capability of other tissues might be addressed utilizing agents that can generate other types of genomic lesions, such as cisplatin which causes

intrastrand DNA crosslinks. Such results might then provide a link between in vivo cellular stress resistance and the processes by which aging is delayed in Snell dwarfs.

The results presented in Chapters V and VI may have broad implications about how lifespan is regulated among many different mammalian species, particularly those within the rodent order. In this chapter, we showed, using samples from eight different wild-caught rodents, one laboratory rodent and a bat species, that there were significant correlations between maximum lifespan and cellular resistance to hydrogen peroxide and cadmium. We also found a trend between lifespan and cellular resistance to MMS and heat, though these correlations did not reach statistical significance. Similarly we found that cells from naked mole-rats, the longest lived rodent species, were significantly more resistant to cadmium, MMS, and heat than cells from mice, and were also more resistant to the oxidative stressor, paraquat. However, we found that cells from long-lived animals are not resistant to all of the stresses which were tested; across mammalian species there was no correlation between lifespan and resistance to UV light or paraquat, and we found the cells from naked mole-rats were significantly more sensitive to the lethal effects of hydrogen peroxide and UV light when compared to cells from mice. Table 7.1 presents a summary of these stress resistance data, together with published data, for comparison, on cells from long-lived mutant mice and from species of long-lived rodents.

The results from these comparative biology studies have several implications. These results are consistent with idea that evolution of long-lived species may require development of cellular resistance to several forms of lethal injury. Also, these results suggest that resistance to some types of cellular injury, such as that generated by cadmium which differed about 60-fold between the longest-lived and shortest-lived animals in these studies, may represent a strong selective force during the evolution of lifespan. In contrast, resistance to paraquat or UV showed no correlation with lifespan in the group of species tested. It might be that resistance to some agents, like cadmium, may be vital to evolution of long life, and resistance to other agents, like paraquat or UV light, are not required. These results might also suggest that resistance to some agents may be vital to extension of lifespan in one group of animals, such as rodents or particular suborders within rodents, while resistance to a different group of stresses may be important in other clades or among animals that have evolved to particular niches. For

example, cells from several mammalian species we tested were far more resistant to UV light than mice, but there was no correlation across lifespan. It may be that these results represent a loss of resistance with selection for a niche wherein these animals may rarely be exposed to sunlight rather than a lack of selection for UV resistance with longevity. Even if resistance to one of these agents is important for longevity in some tissues or organs, we do not know whether resistance to that stress is retained by skin-derived fibroblasts in culture equally among all species. In addition, stress resistance may be unimportant to the direct regulation of aging, but rather may be a consequence of regulation of other lifespan-extending pathways. Finally, results from these comparative biology studies support the idea that resistance to multiple forms of cytotoxic damage may be more indicative of long lifespan than the resistance to any one particular type of damage.

Overall, the data in this thesis are consistent with the idea that the genetic changes that lengthen lifespan, either within a single species or across a group of species, tend to confer cellular resistance to many different forms of cytotoxic stress. These results have also shown that studying characteristics of cells in culture, in particular the regulation of resistance to stress, might further our understanding of how mammalian aging is regulated.

Future Directions

One of the main limitations of the studies presented in this thesis is that all of these experiments have been performed on skin-derived fibroblasts in culture. These cells, while convenient to grow in culture, are unlikely to directly affect longevity or be a factor in the age-related dysfunction of most tissues. At this time, we do not know if other cell types from Snell dwarf mice, or any other long-lived genetic mouse mutant or long-lived mammalian species, are also resistant to agents like peroxide, UV, cadmium, or heat. There is some evidence that CR can increase stress resistance of multiple cell types other than fibroblasts; for instance, DNA repair is increased in cells, including hepatocytes, lymphocytes, and liver and kidney homogenates, derived from CR rodents than in cells from AL control rodents (59-61) and eye lens-derived epithelial cells from

CR mice are resistant to damage induced by hydrogen peroxide (62). There is also some suggestion that several cell types from *p66*^{Shc} knockout mice may be resistant to cyotoxic stress; MEF cultures are resistant to peroxide, UV light, and staurosporine, T-cells from are resistant to dexamethosone, and kidney-derived mesangial cells are resistant to culture in media containing high glucose (63-66). While these data are limited in scope, they suggest that interventions or genetic mutations can affect the cellular stress resistance of multiple cell types, including fibroblasts. Thus, one important future aim will be to develop assays to measure stress resistance in other cell types, like skeletal and heart myocytes, epithelial cells from kidneys and eye lens, and hepatocytes, which might directly contribute to the development of certain age-related pathologies. While much of the methodology for culturing these cells has been extensively worked out for mouse samples, developing methods to culture other differentiated cell types from other mammalian species would require a good deal of effort. Results from such assays might then provide information on both the stress resistance phenotypes of these cells and also suggest causal links between stress resistance, the accumulation of damage, and age-related physiological declines.

It may also be important to determine if long-lived mice exhibit cellular stress resistance in vivo. Discussed in Chapter I, interpretation of whole animal stress resistance can be complicated by the particulars of the complex physiology of mammals; are Ames dwarf mice resistant to paraquat (67) because they have increased oxidative stress resistance in vivo, or do they survive longer because their detoxification of paraquat or progression of lung pathologies differs from control? Addressing questions about stress resistance in vivo will require development of models that measure damage to a specific tissue type or organ following treatment with a stress or toxin. To some extent, experiments of this kind have been attempted using assays that indirectly measure liver toxicity induced by acetaminophen (68) or measure the response of the MEK-ERK kinase signaling cascade in the liver in response to 3-nitropropionic acid (69). However, the interpretation of these studies is still complicated by the possibilities of differences between dwarf and control in the metabolism of these toxins. The skin may be another suitable organ to study in vivo stress resistance. While differences in inflammatory responses may complicate interpretation of long-term results, measuring the damage

induced by treatment of stressors, like UV or other DNA damaging agents, and the kinetics of their repair could be fairly straightforward. It would be of interest then to use many of the techniques presented in Chapter IV to measure the formation and repair of UV lesions in skin cells in vivo. In addition, early reports suggesting Snell dwarf mice are resistant to chemically-induced skin cancer used skin cancer lesions (49;50). Tests using repeated irradiation with UV light to induce skin cancer in mice (70-72) could be used to determine whether Snell dwarf mice are resistant to this agent as well. The use of such an assay may provide insight into both mechanisms of aging and the mechanisms by which cancer development is delayed in dwarf mice.

We know relatively little about the molecular pathways that may regulate Snell dwarf cell stress resistance. A former post doctoral researcher in our lab, Scott Maynard, found that protein expression of HSP-70 is relatively higher in control-derived cells when incubated in conditions of no serum or following treatment with peroxide (73). Amir Sadighi Akha, a research associate in our lab, used a multiplex RT-PCR assay to determine if there were differences between dwarf- and control-derived fibroblasts in the mRNA expression levels of 94 different genes selected by our laboratory members. Within this data set, he found differences in expression between dwarf and control for a number of different genes when cells were incubated with or without serum. Some of these genes were consistently expressed at higher (or lower) levels in dwarf-derived cell regardless of incubation condition. A smaller number of these genes showed genotype specific regulation patterns when the two incubation conditions were compared, i.e. the effect of serum was stronger in dwarf than in control cells. We might then use these data to ask if altering the expression of a gene in a control cell line to be similar to its expression in dwarf cells might increase stress resistance of control cell lines. This could be addressed in cell lines using transfection or RNA interference for those genes found to differ between dwarf and control. The results from these assays may also provide support for analyses of lifespan and cell stress resistance of transgenic mouse models for these genes.

In this thesis, we show similar cellular resistance to stress among Snell and Ames dwarf mice and GHR-KO mice. These mice are also similar in that adults from each mutant have low levels of circulating IGF-I (3-5;67). Others have also suggested that

low IGF-I signaling caused by heterozygous deletion of the IGF-I receptor gene extends lifespan, and mouse embryonic fibroblasts from these mice are resistant to hydrogen peroxide. This suggests that cellular stress resistance may be determined, in vivo, by diminished signaling through the IGF-I pathway. However, it is unknown if the effects of diminished growth factor signaling at specific developmental periods only, such as the progression from juvenile to adult, might have similar effects on lifespan or cell stress resistance as these mutant models. It might be possible to diminish IGF-I signaling in a temporal-specific manner by using transgenic mice with conditional expression of IGF-I binding proteins (IGF-I BP). High IGF-I BP expression at specific times during juvenile development would, in theory, diminish IGF-I signaling during these periods by sequestering the circulating hormone. It might also be possible to modulate growth hormone and IGF-I signaling during these periods pharmacologically. For instance, octreotide is a somatostatin analogue that inhibits growth hormone release and causes a reduction in serum IGF-I, due in part to impaired IGF-I production from many cell types and impairs IGF-I production of multiple cell types (74-77). James Harper, a research associate in our laboratory, is now testing the effects on lifespan and cell stress resistance of octreotide treatments to juvenile mice.

The findings in Chapter IV show that cells from Snell dwarf mice, compared to those from control mice, display an enhanced ability to repair UV lesions. The question remains whether longevity extension through other genetic mutations, dietary interventions, or even across species is always associated with more efficient DNA repair processes. The effects of life-extending CR have been shown to increase the repair of UV lesions in multiple cell types (59-61) and the rate of UV repair has been shown to be positively correlated with maximum donor lifespan in tests of cells from multiple mammalian species with different lifespans (78-83). We have shown that fibroblasts from other long-lived mouse mutants, the Ames dwarf and GHR-KO mice, are like Snell dwarf-derived fibroblasts in their relative resistance to UV light. It would thus be of interest to see if they too show an enhanced ability to remove UV lesions from their DNA relative to their respective controls. This line of investigation could also be extended to other long-lived mouse mutations, in particular the $p66^{Shc}$ knockout mice because MEFs

from these mice, like skin-derived fibroblasts from Snell and Ames dwarf and GHR-KO mice, are resistant to cell death caused by UV light (63).

The data in Chapter IV suggest that nucleotide excision repair processes are augmented in Snell dwarf-derived fibroblasts. As discussed above, it will also be important to test if Snell dwarf mice, in vivo, show greater nucleotide excision repair. How these differences in DNA repair may be a determinant of lifespan may be a more difficult question to address. Transgenic mouse strains lacking different nucleotide excision repair proteins typically show sensitivity to UV light and increased skin cancer rate following treatment with UV light or chemical mutagens; some, but not all, transgenic strains also show an increase in the incidence of spontaneous neoplasia (84). Therefore, a functional nucleotide excision repair pathway seems to be required to maintaining genetic integrity and for preventing the development of cancer caused by exposure to some DNA-damaging reagents. Overexpression of nucleotide expression proteins in cell lines can increase the rate of DNA repair (85) and has been suggested to limit progression of carcinogensis in an in vitro model of human bladder cancer (86). There are no published reports of transgenic mice expressing higher levels of XPC and CSA, two proteins shown to be expressed at higher levels in Snell-derived fibroblasts in Chapter IV. Based on the findings in Chapter IV, it would be of interest to develop such transgenic models to determine if they differ from control in their UV sensitivity, their incidences of spontaneous and induced cancer, and in lifespan.

It will also be important to attempt to determine the upstream regulatory processes by which XPC and CSA are expressed at higher relative levels in dwarf-derived cells. A potential target of these studies might be p53 which is known to play a central role in the recognition of UV lesions (51;54;87). The regulation of XPC expression and activation has been shown to be dependent on the activity of p53 (88;89), as has the activity of the DNA damage binding protein p48 (DDB2) that is thought to localize XPC to sites of UV damage (89;90). Regulation of both XPC and DDB is thought to be important for the recognition of DNA lesions and thus may regulate the rate of global genome repair (91). Also regulated by p53 is Gadd45, which binds to UV-damaged DNA in vitro and is thought to facilitate topoisomerase activity in the presence of histones and thereby increase DNA accessibility for nucleotide excision repair (92;93). It would thus be of

interest to examine the phosphorylation status of p53 in dwarf- and control-derived cells to see if this may be an underlying mechanism of their differential expression of nucleotide excision repair proteins. Following UV irradiation, the activation of p53 caused by UV-mediated DNA damage is thought to be regulated by the stress-response kinase ATR (ATM and Rad3-related) (94;95). Thus, understanding why repair rates differ between dwarf and control following UV irradiation may require careful examination of ATR-mediated stress signaling pathways.

It would also be of interest to see if the cells of Snell dwarf mice, or the mice themselves, differ from control in their ability to repair other forms of genetic lesions. Some have proposed that base excision repair may potentially be more important than nucleotide excision repair in the regulation of aging and age-related diseases such as cancer and neurodegeneration (96;97). Base excision repair is the major pathway responsible for averting the mutagenic and cytotoxic effects of spontaneous hydrolytic, oxidative, and non-enzymatic alkylation DNA damage. In particular, this pathway recognizes and repairs base modifications, such as the oxidation lesion oxo8dG, as well as abasic sites and DNA single-strand breaks. Base excision repair may also play a primary role in the maintenance of a faithful mitochondrial genome (97). There is some suggestion that base excision repair is enhanced in Snell dwarf fibroblasts. In Chapter II, we show that Snell-derived cells are resistant to MMS, a DNA-damaging agent that causes base damage by methylating the N7 position of guanine residues, a lesion repaired exclusively by base excision repair (98). A possible mechanism for this resistance then might be differences between dwarf and normal cells in their ability to remove these lesions. Base excision repair may also protect cells from cell death caused by oxidative stressors, because lethality may in part be due to accumulation of oxo8dG lesions in both the nuclear and mitochondrial genome (99). Thus, determining if base excision repair is more effective in cells from long-lived mice compared to control may then provide a mechanism for their resistance to agents like MMS, and perhaps paraquat and cadmium.

A weakness of our study of cellular stress resistance across multiple mammalian species is a relative lack of species diversity within our collection. It would be of interest to determine whether the pattern of associations noted here between longevity and stress resistance can be replicated in studies that involve a much wider range of rodents. It

would also be of interest to determine if a similar pattern of resistance exists among other closely related groups of species with different longevities, such as among bird species, bat species, or primate species. The sensitivity of naked mole-rats to peroxide, UV light and the mitochondrial poison rotenone suggest that perhaps evolutionary selection pressures unique to an underground niche might affect cellular stress resistance in a manner independent from the relationship between stress resistance and longevity. Thus, it might also be of interest to test groups of underground dwelling mammals compared to closely related above-ground species to test this theory. These data might then show whether similar pathways of longevity are utilized amongst all vertebrates, within only the mammals, or whether different groups of animals have developed different evolutionary pathways in the regulation of lifespan.

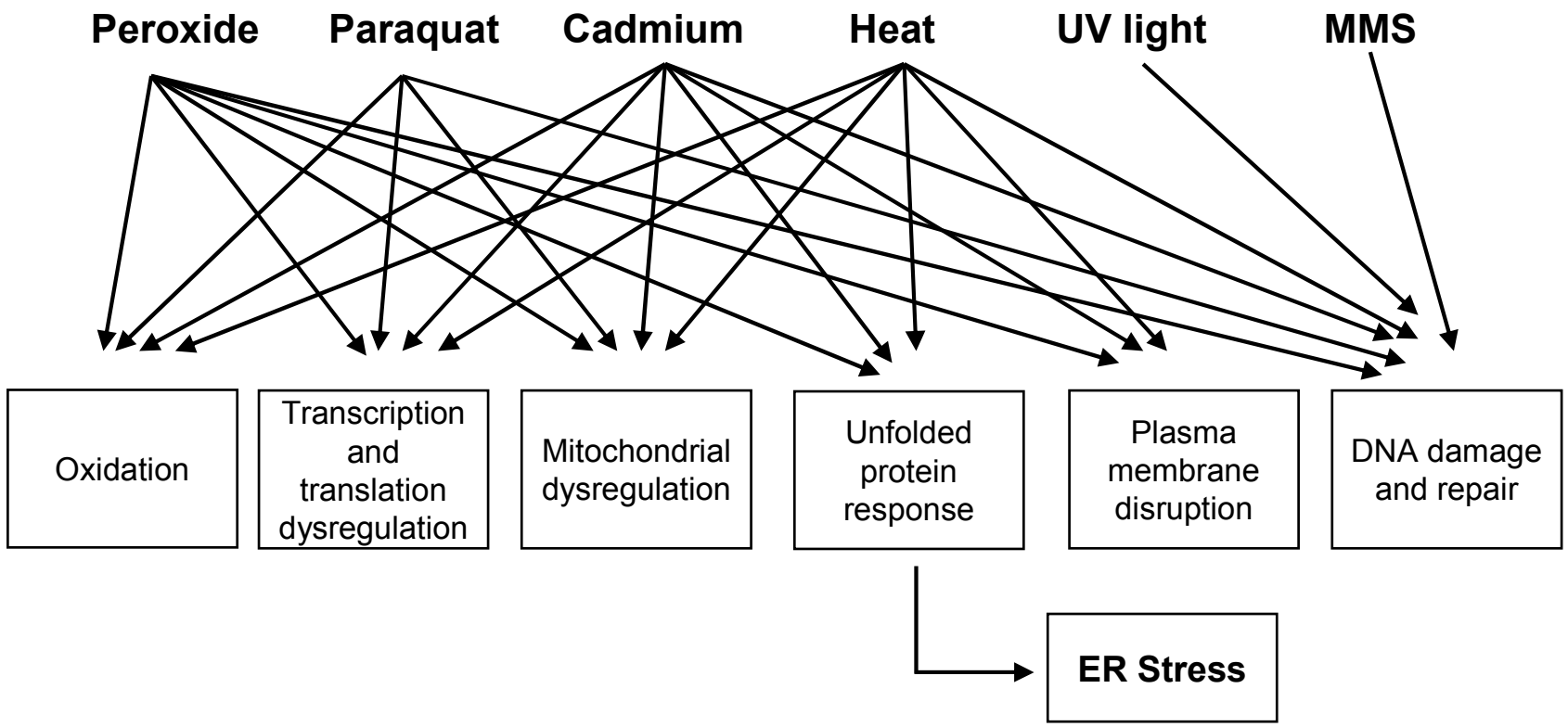

Figure 7.1. Potential pathways of cytotoxicity by agents used in this thesis. Each arrow represents a link found by literatature search between that agent and the mechanisms by which each agent has been shown, or suggested, to induce cell death. In Chapter II, we found that peroxide, paraquat, and cadmium induced cell death, at least in part, through oxidative stress-dependent mechanisms; UV light induced cell death was independent of oxidative stress.

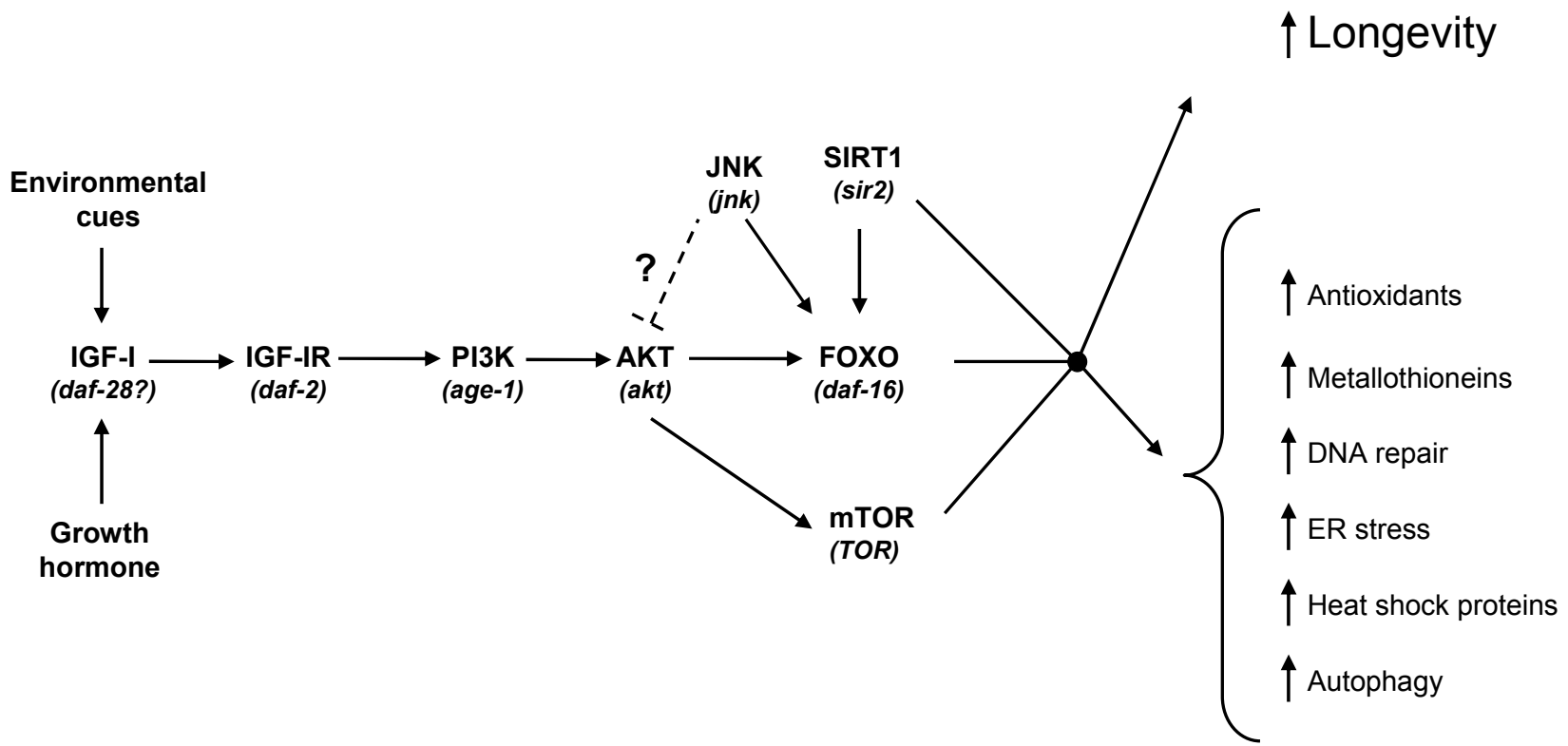

Figure 7.2. Potential pathways regulating lifespan and cellular stress resistance under IGF-I (or insulin-like signal) control. Gene products in bold represent mammalian versions, while italicized gene products represent orthologues from *C. elegans*. Solid arrows represent data from *C. elegans* or mammals showing a relationship between each gene product; dashed line represents an alternate theory of a potential inhibitory relationship.

Table 7.1. Relationship between donor longevity and cellular resistance to multiple agents

	Mean LD50 or ED50 of cells from long-lived rodents relative to control									
Longevity model	**Cad**	**Perox**	**Para**	**UV**	**MMS**	**Heat**	**Glu**	**Rot**	**Thaps**	**Tunic**
Snell dwarf	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓
Ames dwarf	↑	↑	(↑)	↑	↑ [a]	ND	↑ [a]	↑ [a]	ND	ND
GHR-KO	**0**	↑	↑	↑	↑ [a]	ND	**0** [a]	ND	ND	ND
Harper et al., 2007	↑	↑	**0**	**0**	(↑)	(↑)	(↑)	↑	ND	ND
Naked mole-rat	↑	↓	↑	↓	↑	↑	↑	↓	↓	↓

↑ indicates a statistically significant difference ($p < 0.05$) in which cells from the long-lived animals were stress resistant.
↓ indicates a statistically significant difference ($p < 0.05$) in which cells from the long-lived animals were stress sensitive.
(↑) indicates a difference that was not significant, but for which $0.05 < p < 0.1$ for an association between long lifespan and stress resistance.
0 indicates that there was no significant difference between (or among) the groups tested.
ND indicates that the test was not done.

For Snell, Ames, and GHR-KO cell lines, stress resistance is in comparison to cell lines derived from the respective normal littermate animals; data from (1;2;12). The fourth row summarizes results of (100), using simple linear regression to evaluate a data set including 8 species of wild-caught rodents, one species of bat, and a stock of genetically heterogeneous laboratory-adapted mice. (a) indicates unpublished data from James M. Harper.

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