METABOLIC DIFFERENCES IN CELLS FROM LONG-LIVED MICE

by

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PREFACE

The subject of this thesis is aging research, which is a rapidly expanding scientific field of study, due in large part to the increasing number of aged individuals in the industrialized world. With all of the diseases and potential biological fields to study, why did I choose to study aging? This question is especially important to ask a young researcher, since the majority of money allotted to health science research is earmarked for specific diseases (see http://report.nih.gov/rcdc/categories/Default .aspx). In my case, aging has been an interest since my teenage years, when I was one of very few young adults who had a strong sense of my own mortality. It seemed to me that aging, while it may be a consequence of many aspects of life, should not have to be inevitable and should in theory be both relatively preventable and reversible. Years later, I still believe in the theory of my adolescence, only now with a better understanding of how close we are to delaying and/or partially reversing the aging process for people. Finally, the reason for studying aging should not be dismissed as a selfish desire for immortality, but rather a chance to create a healthier, more vigorous aging population, previously termed the "longevity dividend." This means that instead of creating a massive elderly population whose cost to society grows, slowing the aging process would extend the period in life during which people could contribute to society, and compress the time during which people are in a state of morbidity. This would benefit society economically through increased productivity and decreased health care and disease treatment costs. The potential economic savings to society of aging interventions is enough to warrant continued research in aging without even considering the benefit to individuals having more time to live.

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2-DOG	deoxyglucose
ADP	adenosine diphosphate
AFR	ascorbate free radical
AFRR	ascorbate free radical reductase
AhR	aryl hydrocarbon receptor
AL	ad libitum
ALE	advanced lipoxidation end-product
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP-1	activating protein-1
ARE	antioxidant response element
ATP	adenosine triphosphate
BSA	bovine serum albumin
Cb5R	cytochrome b5 reductase
CM	complete media
CNC	cap'n collar
CoQ	coenzyme Q
CR	calorie restriction
CYP	cytochrome P450
DHE	dihydroethidium
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPPP	Diphenyl-1-pyrenylphosphine
DR	dietary restriction
DTNB	5,5'-Dithio-Bis (2-Nitrobenzoic Acid) (Ellman's reagent)

DTT	dithiothreitol
ECF	enhanced chemifluorescence
EDTA	ethylenediaminetetraacetic acid
ED50	effective dose 50%, median effective dose
EpRE	electrophile response element
ERK	extracellular signal-regulated kinase
ER	endoplasmic reticulum
FRTA	free radical theory of aging
FCS	fetal calf serum
FIRKO	fat insulin receptor knockout
FOXO	forkhead box sub-group O
GAPDH	glyceraldehyde phosphate dehydrogenase
GCL	glutamylcysteine ligase
GDP	guanosine diphosphate
GH	growth hormone
GHR/BP	growth hormone receptor binding protein
GHRHR	growth hormone releasing hormone
GHR-KO	growth hormone receptor knockout
GpX	glutathione peroxidase
GSH	glutathione, reduced form
GSK	glycogen synthase kinase
GSSG	glutathione, oxidized form
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	hexokinase
HO-1	heme-oxygenase 1
HPLC	high performance liquid chromatography
HSP	heat shock protein
IGF-I	insulin-like growth factor I
JNK	Jun N-terminal kinase

Keap1	kelch-like ECH-associated protein 1
KO	knockout
KPB	potassium phosphate buffer
KRP	Krebs Ringer phosphate
LD50	lethal dose 50%, median lethal dose
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MB	methylene blue
MCAT	mitochondrial overexpression of human catalase
MDA	malonaldehyde
MEF	murine embryonic fibroblast
MEK	MAPK/ERK kinase
mPMS	1-methoxyphenazine methosulphate
MMS	methyl methanesulfonate
MT	metallothionein
mtDNA	mitochondrial deoxyribonucleic acid
MTOR	mammalian target of rapamycin
MTT	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium
NOX	NAD(P)H oxidase
NQO1	NADH quinone oxidoreductase-1
NRF2	NF-E2 related factor 2
NT	neotetrazolium
PARP-1	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PI	peroxidation index
PI3K	phosphatidyl inositol 3-kinase
PKB	protein kinase B (Akt)
PKC	protein kinase C
PMOR	plasma membrane oxidoreductase

PMRS	plasma membrane redox system
PMSF	phenylmethanesulphonylfluoride
PRL	prolactin
PUFA	polyunsaturated fatty acid
RAS	rat sarcoma
RD50	response dose 50%, median dose
RNA	ribonucleic acid
ROS	reactive oxygen species
RTPCR	Reverse transcription polymerase chain reaction
SD	serum-deprived
SEM	standard error of the mean
SIR	silent information regulator
SOD	superoxide dismutase
Sod1	Copper-zinc superoxide dismutase
Sod2	Manganese superoxide dismutase
tBHQ	tert-Butylhydroquinone
TBO	toluidine blue O
TCA	trichloroacetic acid
TNBT	tetranitroblue tetrazolium
TOR	target of rapamycin
TpMET	trans plasma membrane electron transport system
TSH	thyroid stimulating hormone
TTFA	thenoyl trifluoro acetone
TXRD	thioredoxin reductase
UV	ultraviolet
WST-1	water-soluble tetrazolium
XME	xenobiotic metabolizing enzymes

ABSTRACT

Recent studies of aging have identified several ways to increase the mean and maximum lifespan of a variety of species. In spite of these successes, little is known about the specific mechanisms that control the aging process.

Snell dwarf mice, homozygous for the *dw* mutation at the *Pit1* locus, live ~40% longer than control mice and have slowed development of many age-related pathologies. Previous work established that isolated dermal fibroblasts from long-lived Snell dwarf mice show increased resistance to many toxic stresses as compared to cells from normal littermates. Using a similar approach, we found that dwarf cells were also resistant to the short-term metabolic effects of glucose deprivation and rotenone exposure, measured using a reducible extracellular dye (WST-1). The inhibitory effects were non-lethal and reversible at the doses used, and cellular resistance to low glucose inhibition correlated with resistance to the toxicity of cadmium and hydrogen peroxide among a range of mouse cell lines. In fibroblasts derived from multiple species of rodents, resistance to the effects of rotenone and low glucose medium was correlated with higher species maximum lifespan, consistent with the idea that resistance may evolve with longevity.

Additional work explored the mechanism of resistance to rotenone and low glucose in cells from Snell dwarf mutant mice, focusing on the antioxidant response element (ARE) and its main transcription factor, Nrf2. These studies showed small (~30%) but significant increases in steady-state levels of Nrf2 protein in dwarf-derived cells, accompanied by larger (approximately two-fold) increases in transcription of many ARE-regulated genes. Increased ARE transduction is likely to have wide ranging effects, including increases in the

antioxidant glutathione and activity of the plasma membrane redox system (PMRS), both of which were observed in dwarf cells.

Together, these results suggest that increases in Nrf2/ARE-dependent pathways might contribute to dwarf cellular resistance to the metabolic effects of rotenone and low glucose media, as well as to their resistance to several cytotoxic stresses. Further studies of this pathway may lead to insights as to how the ARE is regulated by dwarf cells, and how ARE activation and stress resistance affect the aging process.

CHAPTER I

INTRODUCTION

The purpose of this thesis, and the goal of this line of research, is to examine the phenomenon of aging. For the context of this work, aging can be defined as a process that converts young, healthy individuals into old, frail individuals with an increased risk of disease and mortality (1). Anecdotal evidence tells us that aging is inevitable and many of the symptoms of aging are visually recognizable with no extra scientific information. Scientific evidence tells us that the aging process affects most if not all cell types, tissues and organs of the body (2). However, despite the ease with which anyone can distinguish an aged individual from a young one, little is known about the specific process(es) that cause aging.

Theories of aging

The definition of aging as a process is important because it looks at aging not as a comparison between young and old, but as a continual process that converts young individuals into old individuals. This process is generally accepted to be occurring, but the scientific mechanism behind it is a hotly debated topic in scientific fields. It is likely that many of the "theories of aging" have some truth to them, in that they describe part of what goes wrong when organisms age, but none thus far have explained how nature can create organisms that live anywhere from hours to centuries, and still seem to age in a way that many parts of the organism begin to fail during a relatively short period of time (2).

Many theories of aging have some rooting in the free radical theory of aging (FRTA). Following an important work by Gershman et al. that suggested that

oxygen free radicals were important for cellular damage (3;4), in 1956, Denham Harman proposed that aging results from progressive damage incurred from the normal production of free radicals in metabolically active cells (5). The accumulation of damage is thought to add up over time and eventually cause the deterioration observed in aged organisms. In the 50 years since it was first proposed, the FRTA has been modified and challenged, (6) but not disproved. One important modification was finding the most commonly produced free radical molecule, superoxide, and its major producer, the oxidative phosphorylation pathway in mitochondria (7). This finding led to the mitochondrial theory of free radical aging, which suggests that the mitochondrial production of ROS leads to accumulating damage and causes age-related deterioration. Some postulate that more specifically, damage to mitochondrial DNA from nearby free radical production leads to dysfunctional mitochondria, further increasing free radical production and beginning a vicious cycle (6) that continually damages the rest of a cell. This theory is supported by various mitochondrial mutator models, where increased mitochondrial mutations cause some age-related problems early in life (8;9). It is also supported by the calorie restriction model of longevity, where decreased food intake leads to lowered production of reactive oxygen species (ROS) and leads to an increase in lifespan in nearly every species tested (10-14). Unfortunately though, the mitochondrial free radical theory of aging has been difficult to prove mechanistically, because difficulties in measuring short-lived ROS as well as the variability of results attempting to mitigate ROS damage through antioxidant treatment, ROS prevention, and mitochondria DNA protection leave the role of mitochondrial theory of aging unclear (Reviewed in (6)).

Significant reports have also been published that contradict the FRTA. Some of these reports involve the overexpression of ROS detoxifying enzymes (discussed further below), and their general lack of an effect on longevity in mammals. These results are confounded somewhat by potentially incorrect assumptions that ROS are necessarily decreased in animals that have increased antioxidant enzymes (15). However, the frequency that an antioxidant intervention, either by

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genetic manipulation of dietary supplementation, has led to an increase in lifespan, is very low, casting doubt on the prominence of the FRTA's role in aging (reviewed in (4)). Thus, work on aging and the FRTA should be understood with the caveat that while reducing free radicals and their associated damage is likely to be an important of the mechanism to slow aging, it is unlikely by itself to be sufficient.

A popular alternative to the FRTA is the somatic mutation theory of aging (16). This theory, which is related to the similar but distinct telomere shortening theory of aging (17), suggests that progressive damage (mutations) to the genome increases over time, and eventually the mutational burden reaches a detrimental level and causes increased frailty. The telomere theory of aging suggests instead that progressive shortening of the ends of DNA, caused by the end replication problem (18) and oxidative stress (19), causes similar genomic instability and increased disease. These theories both hinge on genomic instability as the main cause of aging, and are consistent with the FRTA in that much of the damage to DNA (including telomere shortening) may be caused by free radical damage to either the DNA directly or to the proteins responsible for DNA repair and replication (20). This theory is supported by evidence that mutational load increases with age (reviewed in (21)), but fails to explain why many age-related diseases do not necessarily show mutational causation (22). In addition, the theory does not explain why, while loss of DNA repair proteins can cause decreased lifespan (reviewed in (23;24)), increases in DNA repair have only been correlative in that longer lived species tend to have better DNA repair systems, and have not been reported to increase the lifespan of model organisms. This finding suggests that DNA repair is necessary for health and longevity but unlikely to be the rate-limiting step in aging (16).

Another popular theory of aging is the altered proteins/waste accumulation theory (reviewed in (25)). This theory suggests that while damage to proteins due to ROS and other damaging reagents is inevitable, it is the loss of ability to turn

over damaged proteins over time that causes age-related declines. Important protein and organelle recyclers, including proteasomes and lysosomes, are usually able to efficiently degrade damaged proteins and organelles, but some damaged molecules remain, building up over time and increasing cellular "waste." This theory is consistent with the FRTA in that much of the damage occurring to macromolecules over time may be caused by free radicals, but suggests that the efficiency of degradation decreases with age, exacerbating the problem by causing a buildup of damaged proteins in the cell.

In his attempt to catalogue and classify theories of aging, Medvedev (26) counted more than 300 theories espoused as the theory of aging. As noted above, some of these theories have distinct features, and overlap in other ways, leading to the likelihood that multiple theories have some level of validity. Recent papers have begun to integrate multiple theories into models to attempt to more completely describe, or "network," aging theory (27). This thesis work focuses mainly on a modification of the FRTA, enhancing it to encompass non-free radicals, resulting in the oxidative theory of aging ((20), reviewed in (4)). It does not, however, intend to dismiss other theories out of hand; rather attempting to utilize and expand upon the oxidative theory of aging to interpret a variety of data.

The oxidative theory of aging, like the FRTA, posits that an accumulation of damage from oxygen radicals and other reactive oxygen species eventually causes many of the phenotypes associated with aging (reviewed at length in (4)). Muller et al., suggests that a "weak" version of this theory is well established, in that there is definitely a correlative relationship between oxidative damage and age-related dysfunction (28). However, the "strongest" version of the oxidative theory of aging, which implies that lifespan is determined fully by oxidative damage, remains controversial due to conflicting evidence and difficulty interpreting many results. This is because the strongest version of oxidative theory requires proof of the assumptions that a) oxidative damage causes some of the defects seen in old people and animals (weak version), b) the rate of

accumulation of oxidative damage differs between long-lived and short-lived individuals, or species, and c) differences in oxidative damage explain many of the differences in aging rate between individuals or species. While each of these assumptions is testable, the current difficulties in proving or disproving the oxidative theory lie mainly in the interpretation of so-called "interventionist" studies, which attempt to modify oxidative damage and observe resulting changes in lifespan. These studies are often either unsuccessful in mitigating oxidative damage and/or cause other unforeseen problems that affect lifespan as well. A brief history of these approaches is reviewed by Muller *et al.* (4). A more detailed analysis of some of these attempts is given below.

Models of aging

Many of the advances in the aging field have come from the use of animal models of longevity. Most of the following research utilizes a single model of animal longevity, the Snell dwarf mouse, in order to investigate the aging process. The following is a brief overview of aging models, with attention given to their relationship to the oxidative theory of aging and the important longevity pathways they describe.

Yeast

The first published use of *Saccharomyces cerevisiae*, or budding yeast, for an aging study appeared approximately 50 years ago (29), defining replicative lifespan as the number of daughter cells produced before senescence (reviewed in (30) and (31)). It initially seemed odd that a single-celled organism such as yeast would age and have a finite number of replications, or buds, but initial studies were followed with extensive proof that replicative cycles and not time between buds, was key to yeast lifespan (32). More recent work has aimed to measure the amount of time yeast can remain viable in a post-replicative state, called chronological life span (33). This model, together with the replicative

model, attempts to measure aging in the two main somatic cell types of most multicellular organisms, proliferating and post-mitotic cells. After more than fifty years and hundreds of published papers later, yeast is established as possibly the simplest model of eukaryotic aging, because of the ease of genetic modification and lack of complexity involved (30). However, the ability to translate yeast aging into aging of "higher" organisms is debatable, as both the evolutionary divergence and the increased complexity in higher organisms makes yeast studies difficult to apply more broadly. At best, yeast aging may recreate some of the conditions for generic types of cells in higher creatures, but many of the results may also be peculiar to yeast aging.

The ability to easily manipulate the genetics and the environment of yeast has allowed for the discovery of genes and treatments that are important for yeast aging (reviewed in (31)). Initial studies on how genetic modification could affect yeast lifespan involved the use of a retroviral human RAS gene, which increased replicative lifespan (34). The RAS gene is an oncogene that was known to prevent cell senescence in fibroblasts, and therefore might be expected to act similarly in another cellular model. Soon, other genetic manipulations for extending yeast longevity were published, including the SIR4 loss of function (35), the UTH1 deletion (31;35), the SIR2 overexpression (36), and a recent unbiased screen which produced 13 verified long-lived mutants (37).

In addition to genetic manipulations increasing yeast lifespan, calorie restriction (CR), sometimes referred to as dietary restriction (DR), has also been shown to positively effect both the replicative and chronological lifespan of yeast (38-41). CR as an environmental manipulation for aging intervention has been used since at least 1935 (13). A decrease in calorie intake which is not so much as to cause malnutrition has been the most reproducible and consistent approach to increasing longevity across a variety of species (reviewed in (14;42-44). Most often using decreases of 25-50% from *ad libitum* fed animals, depending on the organism, CR delays or prevents many diseases of aging and extends mean and

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maximum lifespan in most, but not all, organisms tested. It is thought that life extension by CR involves a conserved regulatory system that allows animals to divert energy from reproduction to somatic maintenance in periods of food shortage (27). This theory is supported by gene expression data and damage measurement showing that there is an increase in maintenance in calorie restricted animals (45). Hence, CR is the most accepted method of lifespan extension, and is currently being tested in non-human primates and in some small voluntary human populations (46;47). The effect of CR in yeast has been under recent debate, as the amount of glucose used varies 100-fold from lab to lab and causes disparate results depending on the type of restriction (39;40;48;49). This debate, however, has more to do with the genes and pathways responsible for longevity in CR yeast, and less to do with the ability of CR to increase yeast replicative and chronological lifespan, which remains generally accepted (40;41;49).

As previously alluded to, much of the work in this thesis focuses on aspects of the oxidative theory of aging. In yeast, and most other organisms used, this theory can be examined in multiple ways: first, by examining the accumulation of oxidative damage with aging, second; by looking at interventions aimed at reducing oxidative damage and their effects on aging, and third; by examining the correlation between aging and stress resistance (mostly to oxidative stresses). For this third method, stress resistance may be defined as the ability of a cell, tissue, or organism to resist the toxicity of an exogenous or endogenous stress. Underlying this method is the idea, consistent with both oxidative and other theories, that damage accumulation is the primary cause of aging. Therefore, this method assumes that the ability to resist damage, natural or otherwise, is likely to correlate with a decreased rate of aging (50). The technique of examining aging and stress resistance in yeast was established by the Guarente lab (35), using screens for stress resistant mutant strains of yeast to test for increased replicative lifespan. The correlation has since been verified in the opposite direction, showing increased stress resistance of previously

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identified long-lived mutant strains of yeast (51-53). Finally, there is evidence that oxidative damage plays a role in yeast aging from studies showing segregation of damaged proteins in mother cells (54), studies showing decreased lifespan after loss of superoxide dismutase (55), a key detoxification enzyme, and studies showing that protein carbonyls accumulate during chronological aging (56). However, some of this work is confounded by work showing that growth of budding yeast under anaerobic conditions shortens lifespan, suggesting that yeast aging can be caused by non-oxidative measures (57).

C. elegans

Possibly the most utilized invertebrate model for aging research is the roundworm *Caenorhabditis elegans (58)*. Similar to yeast, *C. elegans* is a popular model because of its relative simplicity (each adult animal has exactly 959 somatic cells), fast growth, rapid development, short life expectancy, ease of genetic manipulation, and more recently, transcriptional modification by RNA interference technology (59;60). The downside to *C. elegans* aging work is this organism's small size, entirely post-mitotic cell population, and large phylogenetic distance from mammals. Nevertheless, in spite of the initial yeast aging data from 1959, it was in *C. elegans* that the first mutations to extend longevity were discovered (61). This initial work quickly brought to light the first, and still some of the most widely used age modifying genes, *age-1* and *daf-2 (62;63)*. Today, at least 276 genes have been identified to extend longevity at various time points in *C. elegans*, and current work continues to identify genes and pathways involved in the aging process (64).

In *C. elegans*, the relationship between the oxidative theory, stress resistance, and aging is perhaps the most clear of all aging models. The initial *age-1* and *daf-2* mutations can induce worms to enter a diapause state called the dauer (65), causing increased expression of genes that protect the worms from stress

resistance. Even in the absence of dauer formation, these long-lived mutant worms were found to be resistant to a variety of toxic stresses, many of which are oxidative in nature (66-72). Researchers also found that both *age-1* and *daf-2* are homologues of the insulin-signaling pathway in mammals. *Age-1* corresponds to phosphatidyl inositol 3-kinase (PI3K) and *daf-2* corresponds to the insulin or insulin-like growth factor-1 (IGF-I) receptor (73;74). Further work showed that *daf-16*, the homologue to a mammalian FOXO transcription factor, was necessary for lifespan extension and stress resistance of the initial insulin pathway mutants as well as others (75;76) This information linked the insulin-like sensory pathway to stress resistance and aging for the first time, a theme that will be revisited throughout this thesis. Overall, the list of *C. elegans* mutants with increased stress resistance and longevity is extensive (reviewed in (77)), including some non-insulin pathway genes (78;79).

Research on *C. elegans* has also examined the relationship of different forms of stress resistance to each other and to aging (80;81), showing that strains selected for resistance to one stress tended to be resistant to several stresses and tended to be long-lived. This work suggests that stress resistance pathways, some of which involve disparate types of stress (i.e. UV irradiation's DNA damage and paraquat's ROS production) may have similar mechanisms of upregulation, and may affect lifespan. Other data suggested that the process of hormesis, by which young animals are subjected to mild stress, can increase stress resistance later in life and increase lifespan (82-84). Several well-known studies on calorie restriction in *C. elegans* use distinctly different methodologies that leave much of the data difficult to interpret (reviewed in (85)). Importantly however, all of the CR techniques have produced some sort of lifespan extension, and at least some have provided data suggesting that stress resistance or antioxidant defense is increased as well (86;87).

Drosophila

The last of the popular invertebrate models for aging research is the fruit fly, *Drosophila melanogaster*. *Drosophila* is a useful model because, like yeast and worms, it is genetically understood, easy to manipulate, short-lived, and easy to maintain. Unlike worms, it provides a model organism with both replicative and post mitotic cells, but, like other invertebrate models, remains phylogenetically distant to mammals. Most of the initial aging work in *Drosophila* used artificial selection for lines of flies with increased longevity (88-91). By selecting for late-life reproduction, researchers were able to isolate flies with increased mean and maximum lifespan, later showing that the flies also tended to be stress resistant (90;91). Further work measuring oxidative damage showed that long-lived flies had decreased protein carbonyls and lipid peroxidation (88;89), consistent with the previous theory on oxidative stress, stress resistance and aging. However, interpretation of these results was complicated by the problem that in *Drosophila*, mating drastically alters the lifespan of female flies (92). Because of this problem, *Drosophila* researchers have turned to genetics to manipulate the lifespan of flies. Random mutant screening has generated mutations in genes such as *Methuselah (mth) (93)* and *I'm not dead yet (Indy) (94),* both of which also caused an increase in resistance to some lethal stressors (93;94). Despite this, very few age-related genes have been found in *Drosophila* by random screening, and therefore directed mutagenesis is more favored in the field.

Studies in *Drosophila* patterned after findings in *C. elegans* have tested the insulin-like signaling pathway for its importance in both lifespan and stress resistance. Mutation of the insulin receptor (*InR) (95),* and the insulin substrate (*chico) (96),* as well as the loss of cells that produce *Drosophila* insulin-like peptides (97) all increase longevity in flies. These mutations are somewhat mixed in their ability to confer stress resistance, as no resistance to heat or paraquat induced stress was found in *chico* flies (96), whereas resistance to heat and paraquat was found in the insulin-like peptide depleted flies (97). However, decreased heart aging has been noted in the *InR* and *chico* mutants (98). Importantly, the results on the insulin-like pathway in *Drosophila* validated many

of the previous longevity results in *C. elegans* in a phylogenetically distinct species.

Results of attempts to increase *Drosophila* lifespan by directly preventing oxidative stress have had mixed results (4). Studies on overexpression of superoxide dismutase (SOD) provide results suggesting increased (99;100), decreased or unchanged lifespan (101), with potential toxicity (99). These experiments did little to test the effects of altered SOD levels on oxidative damage, however, and the question of how overexpression of SOD actually affects oxidative damage with aging in flies is still in debate (4). Some suggest that the results on SOD knockout mice (*Sod1, Sod2* or both) are more informative, as they showed that the deleterious effect of loss of SOD (102;103) accelerated the time-course of an age-related marker (104). They also showed a dose-response relationship between SOD and lifespan (105;106), supporting oxidative theory in so much as oxidative stress can shorten lifespan (4). Similar experiments using exogenous antioxidant treatments on flies have proven inconsistent and difficult to interpret (reviewed in (28;107)), due to lab to lab variation. Other genetic studies using overexpression of a damage repair enzyme (MsrA) (108) and a crucial antioxidant producing enzyme (glutamate cysteine ligase)(109) have increased stress resistance and lifespan in flies, further supporting the oxidative theory (4). Researchers have shown that increased expression of heat shock proteins (HSPs), which are important for response to oxidative and other types of stress (reviewed in (110)), can increase the lifespan and the stress resistance of flies (111;112). Finally, CR has been tested in *Drosophila* to determine its affect on lifespan and stress resistance. The results are mixed, in large part due to differential methodologies (113;114), changes in reproductive capacity, difficulty in measuring actual food intake, and the possibility that the type of calories in the food may be more important than the quantity ((115), reviewed in (116)). Nevertheless, these CR studies have suggested that CR can increase lifespan and stress resistance in flies (114;117-119).

Collectively, results testing mutations, signal transduction pathways, oxidative damage and stress resistance in invertebrates have strengthened the hypothesis that oxidative stress is involved in aging, but have not proven it to be the sole regulator of lifespan. The work has also demonstrated that pathways one might not initially expect to be heavily involved in aging or stress resistance, such as insulin signaling, may play a prominent role. Therefore, some of the mediators and control circuits that regulate aging and stress resistance in invertebrates appear to have their roots in species that predate the split among flies and worms and mammals.

Mouse models of aging

Most of the research in aging and many other fields of biological sciences has used rodent models. Laboratory mice, rats, and to some extent, other rodents have proven invaluable in their contribution to the knowledge of mammalian genetics, metabolism, disease, and aging. Laboratory mice are useful for aging research because mice are small, relatively easy to house, genetically well-understood, have short generation times, and most importantly, are mammals, which makes them very closely related to humans. This means that work on mouse models is likely to translate in some way to humans, providing the means to test ideas about a variety of human processes and diseases.

Aging research in mice was long dominated by the original model of extended longevity, calorie restriction (CR) (14). More than 70 years ago, researchers showed that CR could increase the longevity of rodents by reducing food intake by approximately 33% (13). The lifespan extension can be up to about 50% in rodents (20), and has been well-characterized in terms of gene expression (120), prevention of a wide variety of diseases [(121-124) reviewed in (14)], and stress resistance.

CR protects rodents from a wide variety of organismal stresses. These stresses include paraquat injection (125;126), increased temperature (127), chronic cadmium exposure (128), kainic acid treatment (129), ganciclovir, cyclosporine A and phenobarbitol (130), to name a few. CR mice also show reduced cancer incidence (131). Studies also show that when cultured with the serum from CR animals, cells show similar stress resistance properties as CR animals (132), and further work suggests that the insulin/IGF-1 pathway may be important for the stress resistance. In contrast, cells cultured from CR mice and cultured with fetal calf serum were found to lose stress resistance *in vitro*, suggesting that the stress resistance is a transient state (133).

Long-lived mutant mice:
Until 1996, CR was the only proven model of extended longevity in mammals. It was in 1996 that the first report of a single mutant mouse with increased lifespan of 20-50% was published (134). The mouse was called the Ames dwarf mouse, and had a recessive mutation at the *Prop-1* locus. Since then, more than 20 genetic mouse models of aging have been identified, and genetically increasing mouse longevity has become a standard approach in aging research.

The Ames dwarf mouse and the similar Snell dwarf mouse have single mutations that cause abnormal development of the anterior pituitary gland, leading to decreases in circulating growth hormone (GH), thyroid stimulating hormone (TSH), prolactin, IGF-1 and thyroxin (detailed further below and reviewed in (135)). The Ames dwarf mouse is approximately 33% the size of controls, with decreased metabolic rate and core temperature (136;137). Ames dwarfs were reported to have a 49-68% increase in mean lifespan and a 20-50% increase in maximum lifespan (134). This lifespan increase was initially viewed by skeptics as an effect of dwarf mice mimicking calorie restriction, but further work showed that CR increased the lifespan of Ames dwarf mice even further (138), suggesting separate, although possibly overlapping, mechanistic pathways (139). The separate pathways hypothesis is supported by the mortality curves. The

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dwarf mutation shifts the entire curve to the right (delayed onset of aging), whereas CR changes the slope of the curve (decelerating aging) (138). The Ames dwarf mutation also could be important in human aging, as some evidence suggests humans with mutated *Prop1* may have increased longevity (140).

Many mutant mouse models of extended longevity involve a disruption of insulin-like/IGF-1 signaling. The 'little mouse' is a mutation of the growth hormone releasing hormone (*Ghrhr*) gene, which leads to decreased growth hormone (1%), circulating IGF-1 (9-23%) and body weight (~66%) (141). Little mice are long-lived (142), but to a lesser extent (23% increase over controls) than the Ames and Snell dwarfs, suggesting that growth hormone and IGF-1 changes can lead to increased lifespan, but that thyroid hormone changes can contribute further (136). A knockout mutation of the growth hormone receptor/binding protein (*GHR/BP*), or Laron (GHRKO) mouse (143), also shows increased lifespan (144). Unlike the other dwarf mice, the GHRKO mouse has an increase in circulating growth hormone, but its lack of ability to respond to it leads to a decrease (10% of control levels) in IGF-1 levels. This results in a phenotype of small body weight (40% of controls) and an increase in lifespan of 50-55% on multiple backgrounds (144;145). Interestingly, unlike Ames dwarf mice, GHRKO mice do not respond to CR by increase in lifespan (146). The authors suggest that this is because GHRKO mice lack the elements required to extend lifespan by CR, rather than because the mechanisms of GHRKO and CR longevity overlap or that CR requires GHR (146).

Other mouse mutant models of longevity include the fat-specific insulin receptor knockout (FIRKO) mouse (147), the insulin-like growth factor type I receptor heterozygous knockout mouse (148), the p66*Shc* knockout mouse (149), and the transgenic Hcrt-UCP2 "cool mouse" (150). Of these mice, only the "cool mouse" and p66*Shc* knockout do not directly involve insulin-like signaling. In the cool mouse, a reduction in core body temperature alone is reported to increase lifespan by 12-20%. These data imply that the decrease in temperature in CR

and in hypothermic dwarf mice may contribute to their longevity. Two recent studies also reported an increase in lifespan in the transgenic mitochondrial catalase overexpressor (MCAT) mouse (151) and the transgenic thioredoxin overexpressor mouse (152). In both cases, overexpressing proteins directly involved in oxidative stress resistance were able to increase lifespan, suggesting that mitigating oxidative damage can directly affect lifespan. As of this writing, several other single mutants have been discovered, but like some of those above, many of them are unconfirmed, and are beyond the scope of this thesis.

Many of the mouse mutants described above are consistent with the oxidative theory of aging. For example, Ames dwarf mice show increased expression of SOD1, glutathione peroxidase, and catalase (138;153;154), are resistant to paraquat toxicity (155), have increased xenobiotic defense gene expression, of which some genes are also involved in oxidative detoxification (156), and show reduced levels of DNA and protein oxidation (157). The increased xenobiotic defense is also reported in the little mice with an increased resistance to a variety of liver toxins (158). GHRKO mice are protected from diabetes induced nephropathy (159) and age-related memory loss (160), but were reported to be more susceptible to paraquat toxicity (161), the only finding that does not support oxidative theory. Other supporting data include the IGF-1 receptor heterozygous knockout, in which long-lived females were found to be resistant to paraquat (148). Furthermore, a substantial amount of work on the p66Shc knockout mouse has come to support oxidative theory. The p66Shc protein is thought to play a role as a stress response target downstream of p53 (162;163). These knockout mice are reported to resist paraquat toxicity, to show less oxidative damage at multiple ages as measured by DNA damage and lipid peroxidation (plasma isoprostanes), and to have embryonic fibroblasts that resist peroxide treatment and UV irradiation (163). They also overexpress catalase, and in culture, exhibit reduced apoptosis when faced with oxidative stress (162;164). The p66Shc knockout mouse also has normal size and fertility, in contrast with many dwarf mice, providing a model of increased oxidative stress resistance

without other defects (139). Finally, many recently reported mutants such as the Klotho overexpressor mouse, type 5 adenylyl cyclase KO mouse, and Surf1 KO mouse have also been reported to show some sort of oxidative stress resistance, as reviewed by Bartke (165), further supporting oxidative theory.

The Snell dwarf mouse

The majority of my work has utilized a single mutant model of longevity, the Snell dwarf mouse. Discovered as a recessive, spontaneously occurring mutation in the laboratory of George Snell, Snell dwarf mice were initially characterized as relatively inactive, sterile mice about ¼ the size of normal mice (166). The mutation was later pinpointed to the *Pit1* locus, and was found to interfere with a protein (Pit1) necessary for the differentiation of the anterior pituitary gland, specifically the cells responsible for GH, TSH, and prolactin (PRL) secretion (167;168). As a result, the Snell dwarf mice are deficient in GH, TSH and PRL, and also in secondary hormones IGF-1 and thyroid hormone ((135;136;169)).

Initial studies of aging in the Snell dwarf mouse concluded the dwarfs were short-lived due to compromised T cell-function, and could be a model of accelerated aging (170). Future investigations have not supported the dwarf's loss of T-cell function, and have suggested that when housed properly in specific-pathogen free conditions and provided cage-mates to stay warm, Snell dwarf mice are actually long-lived (171-174). More recent independent studies have confirmed that Snell dwarf mice are, in fact, long-lived, with a mean and maximum lifespan nearly 40% longer than that of their littermate controls (175-177). The dwarf mouse lifespan studies were performed on multiple backgrounds, and produced evidence for slowing of many late-life pathologies, including joint disease, immune and connective tissue changes, and age-dependent dysfunction of the kidney glomerulus and eye lens (175;177;178). The Ames dwarf mutation, which affects the Prop1 locus, whose product acts upstream of Pit1, leads to a similar phenotype, including extended lifespan, delayed age-related cognitive failure, and diminished neoplasia (134;153;160;179). The mechanism has been

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hypothesized to involve IGF-I, because similar results have been obtained using the knockout for growth-hormone receptor binding protein (GHR-KO), a heterozygous null mutation of the IGF-I receptor, and the knockout of growth hormone releasing hormone (lit/lit mice, mutant for Ghrhr) (145;148;175). All of those models have low IGF-I signaling in common, and coupled with data from other organisms, insulin-like signaling seems to be a plausible factor in the regulation of aging rate in mice. However, the dwarf mice are not simply a model of genetically induced caloric restriction. They are not lean like calorie restricted mice, and more importantly, have been shown to further increase lifespan when they are calorie restricted (145;180).

Because the Snell dwarf mouse has been shown to age at a slower rate than its control littermates, our lab has tried to learn more about the biology of cells from these mice, in hopes of obtaining clues to the connection between hormonal changes and the aging process. We use a design in which we compare cells from young adult mutants and controls, to learn more about what factors might act throughout the life span to slow the aging process. This paradigm focuses attention on factors that discriminate fast-aging from slow-aging mice while both are still in good health; the goal is thus to learn what cellular and organismal traits act, at early ages, to delay the rate at which aging impairs function. In addition, use of young mice has some practical advantages: young mice will show less variability in health and disease status, are less expensive, and easier to obtain than old mice.

Based on work showing stress resistance of mutant worms, previous studies from the Miller lab tested the idea that the increased life span of the Snell dwarf might be accompanied by other changes, such as increased cellular resistance to stress. To test the hypothesis that hormonal differences in Snell dwarfs may cause increased stress resistance, Murakami et al. showed that dermal fibroblasts, cultured from the tails of young, healthy adult dwarf mice (3-4 months old), are more resistant than control cells to various forms of cytotoxic

stresses, including heat, UV, cadmium, paraquat, and hydrogen peroxide (181). These differences are seen in cells that have undergone several rounds of mitosis in culture over a period of 2 – 3 weeks, and must therefore be epigenetically stable even when cells are removed from the mice and grown in an artificial environment. These investigators also noted that fetal bovine serum, typically present in culture media, led to an increase in stress resistance of both normal and dwarf cells, obscuring the differences in stress resistance seen after a 24 hour period of culture in serum-free media (181). It is notable that Pit1, the mutated gene in these Snell dwarf mice, is not expressed in fibroblasts, nor in any other non-pituitary cells (182;183). Salmon et al. then went on to show that dwarf cells are resistant to the DNA alkylating agent MMS, and that similar stress resistance is seen in cell lines from the Ames dwarf mouse and the growth hormone receptor knockout mouse (GHR-KO). This work supports the idea that the epigenetic differences in these cells may result from their chronic exposure to low GH/IGF-I levels *in vivo*, prior to explant for cell culture (184).

Cellular metabolism and redox status

In most cells in most situations, basic cellular metabolism involves taking up glucose through specialized transporters, chemically breaking down the glucose into pyruvate during glycolysis, and using pyruvate in the mitochondria to provide electrons for the electron transport chain, eventually providing the cell with ATP. Unfortunately, by using the mitochondrial electron transport chain and its final electron acceptor, oxygen, cellular metabolism is intricately tied to the production of ROS (185). Studies have shown that the production of ROS by the mitochondria increases with age as respiratory function decreases in human liver and skeletal muscle (186;187). Without a concomitant increase in oxidative defenses, this increase in ROS production could lead to the types of cellular damage associated with many diseases of aging Thus, cells have evolved specific signaling pathways, ROS detoxification mechanisms, and repair pathways to mitigate the detrimental effect of ROS damage.

The main way that cells prevent oxidative stress is by maintaining a redox state such that the intracellular environment is a reducing environment (188). There is a delicate balance between the oxidizing environment outside of a cell and the reducing intracellular environment, and changes in ROS production, exterior oxidation, or intracellular antioxidant levels can shift the balance toward oxidative stress. The resulting oxidative stress damages cellular proteins, lipids, and DNA, and has been implicated in the etiology of many age-related diseases (reviewed in (189)). Researchers have suggested that the balance between antioxidant defenses and oxidant formation shifts during aging, causing an increasingly pro-oxidant environment that damages cells (20;190). The evidence for this includes data suggesting that redox controlled metabolic proteins decline in function with age and in age-related diseases (reviewed in (188)), and data showing that increased oxidative damage is evident in aged animals (190-193). Furthermore, decreases in oxidant production have been observed in long-lived models including dwarf mice and CR (194-196), suggesting that attenuation of oxidative stress plays a role in increased lifespan. However, recent work suggests ROS in cells are not always detrimental, and that ROS signaling plays important roles in a variety of cellular processes (reviewed in (197)). ROS have even been shown to regulate protein function in reversible reactions, often protecting cells from damage or regulating processes based on the type of ROS and the microenvironment in which they are created (188). This means that prevention of oxidative stress-related deterioration of function with aging may not be as simple as preventing ROS formation, but may require maintaining a delicate redox balance inside the cell.

The intracellular redox environment is a widely used term with a broad definition: the sum of the contribution of all the redox couples inside the cell (198). Each redox-related molecule or protein can give or accept electrons, often times acting as a cofactor in a reaction and also acting directly or indirectly as an antioxidant. The most abundant cellular redox systems are the nicotinamide system (NAD(P) and NAD(P)H), the glutathione system (GSH and GSSG), and the thioredoxin

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(TRX$_{red}$ and TRX$_{ox}$). Each one is described below, with special attention to the first two, since the reactants are much greater in cellular concentration than the thioredoxin system.

NAD(P)

Nicotinamide adenine dinucleotide (NAD) and its reduced and phosphorylated species are crucial for cellular metabolism, redox, and survival (reviewed in (199)). Although NAD and NADP are structurally similar, NAD(H) has evolved to function mostly in reactions involving substrate oxidation, whereas NADP(H) functions more in reduction processes (200). These cofactors not only contribute to the cellular redox status by providing a sink for electron donation from cellular metabolism and transfer to other redox systems, but they have been shown to play a role in the activity of crucial cellular functions, some of which have been implicated in aging (201-205). NAD-related molecules are not antioxidants by themselves, but are often involved in antioxidant reactions or in reactions that reduce oxidized antioxidants. Thus, they are thought to play an important, but complex, role in cellular redox homeostasis.

Under normal conditions, NAD is much more abundant than NADH (200). Membranes are thought to be impermeable to NAD, and thus there are separate pools of NAD in the cytosol and mitochondria, where reducing equivalents can be passed but not NAD(H) itself (206). Because of this, and because NAD is a consumed cofactor in a number of enzymatic processes, the synthesis of NAD has gained attention in several areas as being crucial to the maintenance of redox status (199;207). Studies have shown that increased NAD synthesis is important for increasing replicative lifespan in yeast, possibly through a *Sir2* mediated pathway (208-210). Enzyme families that consume NAD include: 1) sirtuins, NAD dependent histone and histone protein acetylases thought important for aging (211), 2) PARPs, a family of enzymes that place poly-ADP ribose moieties (PARs) on proteins, and help regulate DNA repair, gene

expression, cell cycle, and cell death (203;212), 3) ADP-ribosyl cyclases, which generate cyclic ADP-ribose and may regulate NAD concentrations (213;214), and 4) mono(ADP-ribosyl)transferases (ARTs), which mono ADP-ribosylate proteins but whose function is less clear (215). Together, the actions of concentration dependent NAD catabolizing enzymes and NAD synthesizing enzymes creates a delicate balance in the pool of NAD.

The reduced variant of NAD, NADH, is characterized mostly in terms of its donation of electrons to the electron transport chain in the mitochondria. However, NADH and its recycling to NAD plays a crucial role in generating a variety of electron transfer conversions, such as pyruvate to lactate in glycolytic cells, NADP to NADPH through mitochondrial transhydrogenase, and as a donor of electrons to pathways including the plasma membrane redox system (PMRS, described below), in order to maintain antioxidant status in the cell (199). Nevertheless, NADH levels are maintained at a very low ratio to NAD levels, and are often oxidized in order to recycle NAD.

In contrast to the ratio of NADH to NAD, levels of NADPH inside the cell are often higher than the levels of NADP (200). NADP(H) synthesis is inherently tied to NAD levels, since NAD acts as a direct precursor to NADP under the actions of NAD kinases (NADKs) (216). Besides mitochondrial transhydrogenase, enzymes involved in NADPH formation from NADP include glucose-6-phosphate dehydrogenase, the well-studied housekeeping gene (217), isocitrate dehydrogenases (218), and malic enzymes. The role of NADPH in cells relates closely to cell metabolism and oxidative stress. It acts as an important part of antioxidant systems, as an electron source for synthesis of fatty acids and DNA, and as a substrate for NADPH oxidase (described below) (199). Importantly for the research described in this dissertation, NADPH's role in antioxidant systems includes regeneration of reduced glutathione (GSH from GSSG) (219), binding to the hydrogen peroxide disposing enzyme catalase (220), and reducing oxidized

thioredoxin (221). From this perspective, donation of electrons from NADPH is imperative for the maintenance of cellular redox status.

Glutathione

The concentration of reduced glutathione (GSH) in cells can approach millimolar levels, making it the most abundant redox-related cellular molecule by 3 to 4 orders of magnitude (222). Because GSH can form mixed disulfides with proteins or small molecules and is reduced metabolically, the redox state of glutathione is strongly coupled to the state of other redox couples. Hence, glutathione is widely considered the most important redox couple and its decline in concentration and reduction with aging is seen as a major cause of oxidative damage (222). Furthermore, glutathione has functions outside of cellular redox maintenance, including modifying the activity of thiol-dependent enzymes (223), acting as a cofactor for enzymes like glutathione peroxidase (224), binding some metals (225), and acting as a storage sink for cysteine (198). Thus, maintenance of glutathione levels and redox state is crucial for maintaining cellular homeostasis.

Glutathione maintenance occurs mainly through two pathways. The first of these is GSH synthesis, which occurs mainly in the cytoplasm, by the rate limiting ligation of glutamate and cysteine by glutamylcysteine ligase (GCL) and the addition of glycine by glutathione synthetase. Second, GSH is maintained by NADPH dependent glutathione reductase-mediated two-electron reduction of GSSG (222). A loss of GSH due to a shortage in enzymatic activity, paucity of molecular donors, or sustained oxidative stress can by itself cause activation of apoptosis (226). In the absence of apoptosis, loss of GSH can cause an increase in hydrogen peroxide levels and an increase in GSH preventable protein mixed disulfides, leaving the cell exposed to increased damage and without the ability to adapt (227). Thus, the age-related decrease in both GSH and the ratio of GSH to GSSG, which has been shown to decrease from 30-120% with age

depending on tissue (228), could well have wide-ranging deleterious effects on cells, tissues and organisms.

The involvement of altered glutathione redox state with aging has been studied in some detail. As mentioned above, studies show that both GSH and the GSH:GSSG ratio decline with age in many mammalian tissues, suggesting that glutathione redox is disrupted with age (228). Part of this change is attributed to a loss in catalytic function of GCL with age, preventing the synthesis of new GSH in old animals (229). Further work showed that glutathione redox state correlated with life expectancy in mice. The tissues and/or mitochondria of liver, kidney, heart, brain and skeletal muscle of the short-lived senescence accelerated mice (SAM) were found to have a lower GSH:GSSG ratio than longer lived C57BL/6 mice, which the authors suggest may contribute to their shorter lifespan (230). More substantial evidence of the role of glutathione in aging has come from the studies of long-lived models, where CR prevented the age-related oxidizing change in mitochondrial glutathione redox state in the kidney, heart, eye and testis of mice (228). Also, several studies in long-lived dwarf and GHRKO mice have shown that glutathione metabolism is altered in dwarf mice, causing higher levels of both GSH and GSSG, possibly due to increased GCL expression in liver and muscle tissue (231;232). Finally, in flies, over-expression of GCL causes a two-fold increase in GSH levels, and neuronal targeting of GCL overexpression led to a 50% increase in lifespan (109).   Altogether, this evidence suggests that glutathione redox state is likely to be important for aging, probably due to its effect on oxidative stress inside cells.

Thioredoxin

 Thioredoxins are oxidoreductase proteins with various roles in cells (reviewed in (233)). They can be electron donors for specific hydrogen peroxide neutralizing enzymes called peroxiredoxins, directly detoxify ROS and refold oxidized proteins (234). Thioredoxins also alter gene expression by reducing cysteine

residues on transcription factors, acting as cell growth factors and apoptosis inhibitors. Their actions and regulation, which are beyond the scope of this work, rely on a specific protein (thioredoxin reductase) for their redox maintenance and play a secondary role to glutathione and NAD(P)(H) in maintaining cellular redox status (235). Notably, however, mice transgenic for thioredoxin I were found to have an increase in mean and maximum lifespan, suggesting its role may be larger than previously believed (152). Thioredoxins are also members of the antioxidant response element, which is described in detail below.

Cellular stress response pathways

In addition to maintaining a carefully controlled cellular redox status (described above) and a system of oxidative stress resistance (described below), cells have evolved stress response pathways in order to respond to potentially harmful changes in the extracellular or intracellular environment. While these pathways are too many and too detailed to cover here, many of the pathways directly or indirectly link cellular stress response and metabolism. Examples of this include the multiple roles of the proteins involved with glucose metabolism, pathways involved in cell metabolism and stress, such as the PI3K/Akt and mTOR pathways, and heat shock response pathways.

Glucose metabolism is the main energy source for a majority of cell types. Thus, it is not surprising that many glucose metabolism and glucose sensing proteins have evolved roles in other pathways, such as cellular stress response. An example of one of these proteins is hexokinase, which catalyzes the first step of glycolysis, but also plays a role in inhibiting apoptosis (236-238) and coordinating glycolysis and oxidative phosphorylation (239;240). Other examples include LDH, which recycles NAD by converting pyruvate to lactate, but also acts as a transcriptional activator, and GAPDH, which produces NADH during glycolysis and also acts as a transcriptional regulator, apoptotic regulator, and redox sensitive enzyme (reviewed in (241)). Several other enzymes related to glucose

metabolism are also redox sensitive, and the levels of glucose in the cell can determine cell survival (242). Finally, there is a class of HSPs known as glucose-regulated stress proteins (GR-HSPs), many of which are chaperones restricted to the endoplasmic reticulum (ER). They were first observed to be activated under nutrient deprivation, but have since been shown to be activated by many cellular stresses. These molecules can provide a link between nutrient sensing pathways and cellular stress response (243). In summary, glucose metabolism not only provides cells with reducing equivalents and energy, but is intricately tied to pathways of stress resistance and redox sensitivity.

Tying in closely with glucose metabolism, stress resistance, and also insulin signaling are the PI3K/Akt and mammalian target of rapamycin (mTOR) pathways. These pathways have been studied in great detail to understand their role in a number of cellular signal transduction events (reviewed in (244;245)). PI3K is an insulin/IGF-1 responsive membrane protein whose many actions include downstream modification of protein kinase B, or Akt (246). Akt helps to regulate several different pathways, including glucose transport, glycolysis, protein synthesis, lipogenesis, glycogen synthesis, cell survival, cell size determination and cell cycle progression (244). Downstream of Akt are important stress responsive pathways mediated by FOXO proteins (247), and cell metabolism, size and growth pathways mediated by mTOR (248).

Many of these pathways have been implicated in aging models or age-related pathways. Examples for PI3K include CR-mediated increases in catalytic to regulatory subunit ratio (249), a necessary role in growth hormone stimulated release of IGF-1 (250), and a prominent role in stress response initiation (reviewed in (251)). FOXO pathways are necessary for the longevity increase provided by insulin-signaling knockouts in worms and are involved in stress response pathways (252;253). Decreasing the activity of TOR has increased the lifespan of yeast (37;254), worms (78), flies (255), and mice (Harrison, Strong, Nadon, Miller et al., submitted), suggesting that the TOR pathway is likely to

have effects on human aging as well. Interestingly, one of the functions of TOR in yeast is to repress stress response systems; decreasing TOR activity increases stress resistance, supporting previous theory (256). Finally, Akt plays a role linking cell survival and metabolism (236;242;257), and its role in activating the FOXO (258-260) and TOR (261) pathways make its involvement in age-related pathways likely.

Molecular chaperones, including the heat shock proteins, also modulate cellular stress resistance. Heat shock proteins (HSPs) are highly conserved ATP driven chaperones whose main role is to maintain the correct folding status of cellular proteins (reviewed in (262)). These proteins are deemed "heat shock" proteins because they are highly activated by sudden cellular stresses, such as a change in temperature. In addition to their role in helping proteins fold correctly, HSPs protect cellular proteins by covering sensitive sites, and if that fails, HSPs recognize irreversibly damaged proteins and target them for degradation. HSPs contribute to the cellular redox state by activating glucose-6-phosphate dehydrogenase and glutathione reductase, and can activate cellular pathways that produce antioxidants (263). Oxidative stress that causes increased protein damage or decreases in GSH can activate the main HSP transcription factor, heat shock factor-1 (HSF1) (264).

Systems of stress resistance/antioxidants

A variety of enzymes and non-enzymatic mediators help to protect cells from oxidative damage from internal and extracellular sources. These include detoxifying proteins, such as catalase, SOD, glutathione peroxidase, peroxidases, metallothioneins, thioredoxin, and glutathione-S-transferase, as well as organic antioxidants such as ascorbate, α-tocopherol, Coenzyme Q, bilirubin, and albumin (reviewed in (265)) (see Table 1.1 for brief descriptions). The importance of each of these antioxidants is still debated, but interesting work regarding their role in aging has emerged. First, mitochondrial catalase

overexpression has been shown to increase lifespan by 20% in mice (151), and increased catalase activity has been found in tissues of long-lived Ames dwarf mice (154;266). However, conflicting data suggest that long-lived GHR-KO mice and CR mice both express lower levels of catalase than controls (161;267;268), leaving no consistency on the role of catalase in aging. Second, superoxide dismutases (SODs) have been extensively studied for their role in aging. The results suggest that *Sod1* knockouts are oxidatively stressed and short-lived (269), and *Sod2* knockouts are developmentally lethal (270), but increased expression, while effective in flies, has shown no lifespan increases and even some detrimental effects in mice (15). Third, other antioxidant knockouts have had varying effects; a glutathione peroxidase knockout showed no phenotype (271), a peroxiredoxin knockout shortened lifespan (272), and methionine sulfoxide reductase knockouts shortened lifespan (273) in mice. The roles of the specific antioxidants in aging are likely to be redundant in some cases, nonexistent in others, and thoroughly complicated in every case. This has led some to investigate the role of systems of antioxidant defenses, some of which are described below.

The antioxidant response element (ARE)

One of the most potent (in terms of oxidative stress) and widely studied systems of stress resistance is the antioxidant response element (ARE), or electrophile response element (EpRE). First described 20 years ago (274) and soon after characterized based on an enhancer sequence in the rat GST promoter (275;276) and human NQO1 promoter (277), the ARE is a *cis*-acting promoter element involved in phase II detoxification (reviewed in (278;279)). The ARE is distinct from, but can be coordinately regulated with, the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor that activates phase I detoxification systems (280). Phase I enzymes mainly include xenobiotic-metabolizing enzymes (XMEs), such as members of the cytochrome P450 family, which catalyze chemical reactions with foreign toxins (bioactivation),

often generating ROS and toxic intermediates (280). The ARE battery of phase II detoxifying enzymes minimizes the damage from phase I created intermediates by preventing oxidative and otherwise harmful damage to cells (281).

Proteins regulated by the ARE are involved with glutathione synthesis (282;283) and maintenance (284), protein turnover (285), antioxidant expression (286;287), oxidant inactivation (288), NADPH synthesis (289;290), toxin export (289), and inflammation prevention (291). The family of genes (>200) regulated by AREs maintain cellular redox status in the face of a wide variety of toxins and oxidants through protein and lipid maintenance, chemoprevention, and neutralization (279). The specific genes involved are too many to list here (see Table 1 in (290)), but some of the more important proteins to this work are listed in Table 1.2. All of these proteins can play important roles in resistance to oxidative stress, and represent a battery of genes that could make a cell or organism resistant to a variety of toxic stressors.

Shortly after the discovery of the ARE, it was suggested that the consensus ARE sequence was similar to the binding sequence of Activating Protein-1 (AP-1) (275;292;293). While AP-1 turned out not to bind strongly to the ARE, another protein, NF-E2-related factor 2 (Nrf2), was cloned based on its ability to bind the NF-E2/AP-1 repeat in the promoter of the beta-globin gene (294). Nrf2 was further described as a ubiquitous protein whose binding motif (along with the related Nrf1 transcription factor) matches that of the ARE (295). Nrf2 belongs to a family of transcription factors called the Cap'n'Collar (CNC) proteins (296;297). The members of this group have a CNC domain, which binds directly to DNA, and a conserved basic-region leucine zipper (bZip) domain that binds to other proteins containing this domain, mainly thought to be small Maf proteins (297;298). Other members of the CNC family include Nrf1 (299), Nrf3 (300), p45 NF-E2, Bach1, and Bach2 (reviewed in (279)).

Utilizing homologous domains from a variety of species, researchers found a *trans*-activation domain in Nrf2 that was likely to be the binding site of a repressor protein. Using a yeast two-hybrid screen, the Yamamoto group was able to isolate kelch-like ECH-associated protein 1 (Keap1) as the repressor protein that binds to Nrf2 (301). Further work confirmed this interaction *in vivo* and found that Keap1 transcription is similar to that of Nrf2. Keap1 was also found to interact with the actin cytoskeleton (tethering Nrf2 in the cytoplasm), and to contain a ubiquitin E3 ligase activity (302), which targets Nrf2 for proteosomal degradation. In the presence of Keap1, the half-life of Nrf2 protein is less than 20 minutes (303-305), allowing cells to have high transcriptional levels of Nrf2 with low constitutive protein levels (279). The targeted degradation of Nrf2 is also supported by work showing that activators of the ARE increased Nrf2 protein levels without increasing Nrf2 transcription (303;305;306).

Further analysis of the structure of Keap1 and the Keap1/Nrf2 interaction found that Keap1 contains a large number of conserved cysteine residues (27) that are important for Nrf2 binding and activation (279). This finding led to the idea that Keap1 can be both a repressor of Nrf2 and a molecular sensor of oxidative stress, because cysteine residues are particularly sensitive to oxidative stress (279;307-309). Three of these cysteine residues (C151, C273, and C288) were found to be important to Nrf-2 induction by some activators (310-313), while other activators appear to use other mechanisms (314). This has led to a model whereby Keap1 functions as a molecular switch, maintaining low Nrf2 levels when cellular redox status is maintained, and turning on Nrf2 transcriptional activity when levels of oxidative stress exceed a threshold (315).

Nrf2 activation can also occur via cellular signal transduction pathways (316). However, the work studying these pathways has been largely correlative and non-mechanistic, often using inhibitors that affect broad signal transduction pathways instead of describing specific interactions *in vivo* or *in vitro*. Nevertheless, Nrf2 activation has been linked to at least four different cellular

signal transduction pathways with varying degrees of confidence. Each pathway is thought to phosphorylate and stabilize Nrf2, but some have argued that phosphorylation fails to stabilize Nrf2, and may instead change its transcriptional activity (316).

First, Nrf2 is thought to be activated downstream of phosphatidylinositol 3-kinase (PI3K) (317). PI3K is a lipid kinase that is associated with cell survival pathways and growth factor signaling (318;319). PI3K substrates are messengers for Akt kinases and S6 kinases, which are important for cellular growth, survival, and metabolism (320). Studies on Nrf2 and PI3K have suggested that inhibition of PI3K with wortmannin or LY 294002 prevents induction of the Nrf2 signaling pathway (321;322). This work is supported by studies showing that Nrf2 is localized at the membrane prior to its phosphorylation and nuclear relocation, with the potential for PI3K (or PKC) to directly phosphorylate Nrf2 at the membrane (322). It is suggested that PI3K directly or indirectly (through signal transduction) phosphorylates Nrf2, although the site and resulting effects are unknown at this time.

The second pathway implicated in Nrf2 activation is the mitogen activated protein kinase (MAPK) pathway (323;324). The MAPK pathway is a broad stress and survival signaling pathway that is often initiated at the cell surface and involves at least 3 subpathways (325;326). The inference that MAPK is involved in Nrf2 pathways is based on data showing that some Nrf2 activators also increase phosphorylation of MAPK protein ERK1/2 (323), and that inhibition of this ERK phosphorylation by PD98059 prevented induction of ARE-linked reporter activation. Other studies have also correlated decreased MAPK activity with decreased Nrf2 expression, but have not given insights into the mechanisms involved (327). Other data have produced conflicting observations, suggesting that phosphorylation of Nrf2 is unaffected by MAPK inhibitors, leaving involvement of ERK in Nrf2 signaling in debate (328;329). It is notable, however,

that Snell dwarf livers have been found to have differential activity of both MAPK and Akt (PKB) pathways (330).

The third and best-defined signal transduction pathway for Nrf2 activation involves protein kinase C (PKC) (329;331). PKCs are a group of isozymes important for signal transduction from the plasma membrane to the nucleus (332). PKC is a plausible mediator for Nrf2 activation because it responds to extracellular stimuli and may be able to detect changes in cellular redox status (333). The data on PKC and Nrf2 activation show that PKC phosphorylates Nrf2 at Serine 40 *in vitro*, that PKC activation increases ARE activation, and that PKC inhibition suppresses ARE activation. These studies also showed that Nrf2 can be phosphorylated by PKC immunoprecipitates, providing further support for interaction between PKC and Nrf2. Thus, PKC seems the most promising signal transduction pathway of Nrf2 induction, but more information is needed about what signals lead to PKC activation and how phosphorylation affects Nrf2.

Genetic modifications

Work using genetically modified mice has strongly supported the role of Nrf2, and closely-related Nrf1, as the transcription factors for the ARE. Surprisingly, Nrf2 knockout mice were born without an obvious phenotype, suggesting that Nrf2 is not required for development (334). However, the ARE is an adaptive response, and Nrf2 knockout mice show increased susceptibility to a wide range of toxins (reviewed in (335)), illustrating that they are unable to adapt to stress. Further work exposing knockouts and wild type mice to Nrf2 activators showed that Nrf2 can activate a wide range of genes, including antioxidants, transporters, UDP-glucuronosyltransferases, and components of the proteasome (290;336-338). Use of the Nrf2 knockout mouse in formal aging studies has been limited to a study in which it was shown that the resistance to cancer previously observed in CR mice required Nrf2, and that CR extended the lifespan of Nrf2 knockout mice (339). The study did not, however, control for the short lifespan of the Nrf2 knockout, previously reported to be due to hemolytic anemia (340).

Nrf1 is also a transcription factor, isolated similarly to the closely-related Nrf2, but is less well understood (299). It is possible that Nrf1 regulates basal levels of ARE genes while Nrf2 provides an adaptive response to cellular stress. This idea is supported by the relatively high steady-state levels of Nrf1, Nrf1's ability to bind the ARE, and by the fact that Nrf1 knockout mice are embryonic lethal, signaling that Nrf1 is required for development (341;342). Nrf1 has also been found to bind Keap1 *in vitro*, but Keap1 does not appear to affect Nrf1 transcriptional activity in cells (343). Further work has suggested that Nrf1 and Nrf2 may have overlapping but distinct roles in ARE activation (344). In general, the published work suggests there may be separate and redundant roles between the related Nrf proteins, and they both are necessary for proper basal and stress-related ARE expression.

Studies of the Keap1 knockout mouse have been stymied because the homozygous null mutation caused hyperkeratosis in the esophagus and forestomach, leading to death before adulthood (345). This phenotype was accompanied with a strong induction of the ARE, and was rescued by crossing with Nrf2 knockout mice, further supporting the relationship between Keap1 and Nrf2 (345). There is little information about Keap1 heterozygous mice, beyond the observation that these mice are phenotypically normal and had half the expression of Keap1 compared to normal mice (345).

More recently, work in *C. elegans* has shown that the Nrf2 homologue, SKN-1, is required for the increase in longevity during CR (346). Further work showed that SKN-1 was not only necessary for life extension through insulin pathway disruption, but that increases in SKN-1 alone could increase the lifespan of worms (347). However, SKN-1, unlike Nrf2, does not have a binding partner in worms, and thus its increased expression was unrelated to its repression. Recent work in *Drosophila* has also shown that a heterozygous knockout of the fly Keap1 gene increases stress resistance and lifespan in males, further supporting the role of Nrf2 in aging (348). Together, the role of Nrf2 in lower

organisms and its role in stress resistance in mammals justifies further study of Nrf2 transgenics and tissue and time-specific Keap1 knockouts.

Nrf2 activation

The ARE can be activated by a wide variety of compounds, including phenolic antioxidants (275), isothiocyanates (349), heavy metals (350), and heme complexes (351;352). In total, there are 10 classes of Nrf2 activators based on their chemical structures, and the only thing they have in common is chemical classification as electrophiles (353). Of these activators, sulforaphane, a natural isothiocyanate found in broccoli, arsenite, a toxic metal, tert-butyl hydroquinone (tBHQ), a synthetic phenolic antioxidant, and oltipraz, a cancer chemopreventive drug, have been the most widely studied (reviewed in (281)). Among these four compounds, the mechanism of action seems to vary, from prevention of Nrf2 proteolysis by tBHQ to separation from Keap1 and nuclear translocation by arsenite. Little is understood about the exact mechanism of Nrf2 activation except the electrophilic nature of each activator (315). Furthermore, some ARE activators are bifunctional activators of both the XRE (phase I) and the ARE (phase II) (354). These activators include TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (355), and can act either by producing an ARE activating compound when metabolized by CYP proteins, or by AhR activation, which has been found to activate Nrf2 transcription through XREs in the Nrf2 gene (356).

The inference that Nrf2 and ARE play a role in aging comes mainly from studies in lower organisms and the oxidative stress resistance pathway they activate. Nrf2's role in cancer resistance has been more widely studied, showing that Nrf2 activators are chemopreventative agents and that Nrf2 activation protects some tumor cells from chemotherapy (reviewed in (315)). In age-related pathways, Nrf2 has been shown to control insulin receptor signaling in one cell type (357), and resveratrol, sometimes claimed to have anti-aging effects, has been found to activate Nrf2 (358). Also, evidence has shown that Nrf2 steady-state levels decrease with age (327), and that Nrf2 can play a protective role in many age-

related diseases, including Alzheimer's disease, Parkinson's disease, cardiovascular disease, emphysema, and macular degeneration (reviewed in (359)). To date, the data suggest that the Nrf2/ARE system is a strong candidate for age-related research, and future work will likely elucidate the role of the Nrf2/ARE system in aging pathways.

Plasma membrane damage and aging

The importance of cellular membranes to maintenance of function and homeostasis is generally accepted (360). The cell membrane is made up of lipids and other amphipathic molecules held together by non-covalent bonds (361). Cellular membranes serve to separate the cell from the outside environment as well as to separate different organelles from each other, regulating the movement of a wide variety of molecules. Membranes also provide a matrix for many cellular processes and are important for both anabolic and catabolic processes. The requirements of intracellular and extracellular metabolic and cell signaling force the plasma membrane to be a responsive and dynamic cellular feature (362). Thus, the ability of cellular membranes to maintain proper function greatly depends on the ability of cells to maintain their membrane composition and infrastructure (360).

The idea that alterations of the plasma membrane modulate aging has gained acceptance in recent years (360). Some have even suggested that the loss of membrane integrity is the root cause of the aging process (363). Much of the current theory on plasma membranes and aging is based on evidence that the composition of cellular membrane varies distinctly between species and relates to metabolic rate and lifespan (reviewed in (364)). Hulbert *et al.* argue that lipid peroxidation products are reactive and damaging and that fatty acids have differential susceptibility to peroxidation to suggest that membrane composition could hold a key to lifespan (364). The argument is supported by data showing that increases in lipid damage are observed in a variety of species during aging

(see tables 5 and 6 in (364)). Notably, increases in lipid hydroperoxides and their breakdown products (MDA), the polyunsaturated fatty acid content (PUFA), and the concentration of hydrocarbons in expired air have been observed with aging, correlating with a decrease in membrane fluidity.

Discussion of the role of membranes in cellular integrity and aging requires some background information on lipid composition and damage. Membranes are composed of a variety of phospholipids, sterols, and proteins necessary for the creation of membrane fluidity and dynamics (361). Most damage to membranes involves the attack of phospholipids by reactive oxygen species (ROS). However, the susceptibility of membrane lipids is not identical between lipids and depends on reactivity, or peroxidizability of the fatty acid (360). Furthermore, the impact of the stress will depend on the type of ROS, the lipid target, the anti-oxidant defense systems nearby, and repair and removal processes (360).

Susceptibility of lipids to oxidative stress depends on their chemical properties (364). PUFA residues, for example, are extremely sensitive to oxidation, and can form lipid hydroperoxides that lead to long free radical chain reactions, damaging large portions of the membrane (361). This occurs because the carbon radicals generated in the interior of the membranes usually react with oxygen dissolved in the membrane, creating peroxyl radicals that react with other lipids or proteins nearby, damaging the membrane and possibly the interior of the cell (365;366). Lipid hydroperoxides are also more hydrophilic than normal fatty acids, and migrate to the membrane surface to interact with water, disrupting the membrane, increasing rigidity, and sometimes causing a loss of integrity (360). The susceptibilities of various fatty acids to peroxidation have been calculated and relative susceptibility to peroxidation damage can be expressed in a peroxidation index (367). Lipid peroxidation causes formation of hydroperoxides, endoperoxides, and various intermediates, many of which have longer half-lives than free radicals, and are thus measurable (361;365). Some of the products of lipid peroxidation can also react with proteins and DNA, forming adducts and

cross-links called advanced lipoxidation end-products (ALEs) (368). These products accumulate and form lipofuscin, a nondegradable fluorescent pigment found in damaged or aged cells (369). Together, the damage caused by 'lipoxidative stress' is a relatively irreversible damage which lowers the integrity of cellular membranes and damages intracellular proteins and DNA as well.

One way to attenuate damage at the plasma membrane is to repair, replace, or degrade the ALE modified molecules (364). This can be achieved in part by glutathione peroxidases (GPX), which can reduce hydroperoxides to fatty acid alcohols after hydroperoxides are released from the membrane, or in the membrane by a GPX variant known as phospholipid hydroperoxide glutathione peroxidase (PHGPX) (370). Constant lipid remodeling by phospholipases and acyltransferases can also restore membrane integrity (371). Downstream lipoxidation species, such as carbonyl adducts, can be neutralized by several enzymatic reactions or by glutathione conjugation (361), and may also activate the antioxidant response element (described above) (279). Lipid turnover and repair systems act together to prevent oxidative stress from damaging the integrity of the plasma membrane.

As mentioned above, many measurable indices of membrane damage and damage susceptibility increase with age (364). There is an inverse correlation between membrane saturation (peroxidation index, or PI) and maximum lifespan (372;373). A change in PI is also thought to provide a mechanism for increased lifespan by CR (360), in conjunction with decreased ROS production and increased antioxidant maintenance (374-376). CR is known to prevent age-related changes in membrane unsaturation and lipoxidation-derived damage in multiple cell types and species (360;377). Membrane antioxidant defenses (described below), including protein expression and coenzyme Q levels, are also known to decline with age, but are increased significantly by long-term CR treatment (374;375;378). Increased antioxidant defense and modified fatty acid

composition leads to a decrease in membrane oxidative damage with aging (374;375;378;379).

Protection and redox regulation at the plasma membrane

In order to prevent the damage that can occur in the plasma membrane, cells have evolved specific antioxidant systems to protect the integrity of the membrane (361). This protection is mediated mainly through a group of proteins and antioxidants called the plasma membrane redox system (PMRS) (380), once known as the plasma membrane oxidoreductase (PMOR), and occasionally still referred to as the trans-plasma membrane electron transport system (TpMET) (381). Plasma membrane redox systems have been found in all cell types, but their exact components and functions have not been fully described (382;383). The PMRS (reviewed in (380;384)) consists of documented proteins, such as NADH quinone oxidoreductase-1 (NQO1), cytochrome b5 reductase (Cb5r), and some NAD(P)H oxidases (NOX's), as well as documented activities and unknown protein components. The final members of the PMRS are the antioxidants, consisting of α-tocopherol (Vitamin E) and coenzyme Q in the plasma membrane, and ascorbate and glutathione outside and/or inside the cell.

The main purpose of the PMRS appears to involve the protection of the plasma membrane from the lipoxidative damage described above. Most membranes contain approximately one tocopherol molecule per thousand lipid molecules (385). Tocopherol is known to scavenge lipid peroxyls and reduce them to hydroperoxides, preventing chain reactions (365). Oxidized tocopherol is then recycled into its reduced form by either coenzyme Q (CoQ) (ubiquinol) or ascorbate, each of which is subsequently re-reduced by enzymes in the PMRS. Coenzyme Q is a particularly important membrane antioxidant because it is synthesized by all examined cells and has specific NAD(P)H reductases responsible for maintaining/restoring its reduced status (361). Also, increasing coenzyme Q levels, specifically CoQ10, are suggested to help increase the

lifespan of lower organisms and may be involved in the mechanism of stress response (386-388). Collectively, CoQ acts to stabilize plasma membranes, regenerate antioxidants, and regulate the ceramide-dependent apoptosis pathway (376;389).

The proteins responsible for maintaining plasma membrane antioxidant status include two that are relatively well characterized: NQO1 and Cyb5R (390-392). NQO1 is a cytosolic protein, also functioning away from the membrane and transiently attached to the membrane under certain circumstances. Cyb5R is permanently attached to the membrane on the cytosolic side (393;394), where it has both innermembrane and transmembrane activities (381). The expression of these proteins is known to be affected by oxidative stress, unsaturated fat intake (395), vitamin E intake, selenium intake (376) and serum withdrawal (396). As mentioned above, PMRS enzyme activity and antioxidant (CoQ and α-tocopherol) levels decrease with age in rat brain and liver, but this decrease is prevented by CR (374;375;378). The electron donors for the PMRS are not completely agreed upon. Many researchers suggest NAD(P)H to be the main source of electrons (393;397), but there is evidence in some cell types that ascorbic acid and GSH donate electrons into and across the membrane, and NAD(P)H is used mainly to reduce the resulting ascorbate free radical and GSSG (381;394). It would seem, by the undefined nature of the proteins of the PMRS, that NAD(P)H, ascorbate, and glutathione might all make some contribution toward PMRS electron donation, depending on the cell type and metabolic situation.

Another proposed role of the PMRS involves its ability to regulate the redox status of cells (398). This involves the passing of electrons, mainly from NAD(P)H, from the cytosol across the cell membrane in order to recycle NAD(P) for further use. This can be important in the maintenance of the NAD(P): NAD(P)H ratio, which is essential for a number of processes in the cell, both metabolic and otherwise (reviewed in (393;399)). Much of the recent work on

PMRS activity has utilized mitochondrial gene-knockout cells (ρ^0), as the loss of mitochondrial function forces cells to upregulate the PMRS due to a lack of an oxidative phosphorylation pathway (400). This, along with other work in cells with active mitochondria (401), has shown that cells pass electrons across the membrane to extracellular acceptors in order to regulate intracellular redox status. While the PMRS was originally identified as a system by which cells reduced extracellular ferricyanide (402), there are now several known extracellular acceptors of PMRS electrons (detailed below) (403;404). Experimental evidence generally suggests that the PMRS may have evolved to adjust cellular (or cytosolic) redox state while maintaining the plasma membrane antioxidants and protecting the cell.

The least well understood PMRS function is its role in cell signaling and cell damage (380;405). Through production of superoxide, surface NOX proteins can create a growth signal to nearby cells. This would seem to antagonize the other PMRS functions in that it would promote oxidative damage, and has been studied best in neutrophils, whose activated NOX proteins produce an oxidative burst used to kill infecting species (398;406;407). However, even non-neutrophilic cells can produce superoxide and peroxide, causing activation of membrane tyrosine kinases (381). An age-related increased in NOX activity has been observed in the cardiovascular system (396), and it is thought that disruption of CoQ electron transport at the membrane can provide a major source of ROS, similar to the mitochondria (193;396). This supports a role for the PMRS in both preventing and promoting oxidative damage, thus its regulation may be important for aging processes.

Use of redox-sensitive dyes as indicators of cell viability

Exogenous dyes are often used as indicators of cellular growth, viability, and metabolism (403;404;407-411). Much of the work in my thesis has utilized a dye, water-soluble tetrazolium-1 (WST-1), to measure metabolic functions and

viability in cells. In some protocols, WST-1 can be used to measure cellular viability after cytotoxic stress assays (181). With other methods, reduction of WST-1 can provide an index of PMRS activity (reviewed in (403)).

Tetrazolium dyes are salts that can be reduced by living and metabolically active cells, causing an increase in color (403). Their chemistry depends on a positively charged tetrazolium ring containing four nitrogen atoms, surrounded by aromatic phenyl moieties (403). Upon reduction, the tetrazolium ring is disrupted, causing transformation into a brightly colored formazan product. This chemistry is the basis of all the tetrazolium dyes, which differ in the chemical structures surrounding the tetrazolium ring (403). The original members of these dye families were triphenyl tetrazolium chloride (409), ditetrazolium salts such as neotetrazolium (NT), nitroblue tetrazolium (NBT), and tetranitroblue tetrazolium (TNBT), as well as monotetrazolium salts such as 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT). These early dyes (reviewed in (412)), tended to be positively charged, and thus were taken up by cells and reduced by a variety of intracellular organelles, including mitochondria and lysosomes. The ditetrazoliums are important for use in tissue studies because their formazan products bind to tissue proteins, whereas monotetrazoliums have been more useful in cellular endpoint assays because they form large crystals that kill cells and spread across tissues (403). The ability of monotetrazolium dyes to enter cells and provide viability and intracellular redox measurement led to their wide use in microplate assays (413;414).

The newer, second generation tetrazolium dyes have added negatively charged sulfonate groups to the outer phenyl moieties, producing soluble analogues that do not damage cells (403;415). These dyes, including XTT, MTS and WST-1, eliminated the need for solubilization steps and allowed for their use in assays that depend on continuous monitoring rather than assessment at a single endpoint. Because these salts are strongly negatively charged, however, they fail to cross the plasma membrane to enter cells (416). This has led to some

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confusion as to what these dyes actually measure (the manufacturer states that mitochondrial dehydrogenases are responsible for their reduction), but has also led to their increased use to measure electrons passing through the plasma membrane via the PMRS.

WST-1 is sold commercially in a kit including an intermediate electron acceptor, 1-methoxyphenazine methosulphate (mPMS), and is often used in tests of cellular viability. However, because of its inability to cross the membrane (398;408), WST-1 can also be used to measure PMRS activity in live cells, and has been used extensively in assays for the PMRS (406;415;417). Assessments of experimental protocols that depend on measurement of WST-1 reduction thus require independent determination of whether the experimental conditions do or do not lead to cell death. Studies of the mechanism of WST-1 reduction suggest 1) that WST-1 competes with molecular oxygen for PMRS electrons (418), 2) that superoxide is indirectly involved in the reduction of WST-1 (408;418), 3) that in the presence of mPMS, WST-1 can be readily reduced by electron donors such as NAD(P)H and GSH (403), 4) that CoQ is probably involved as an electron donor (398;418), and 5) that WST-1 reduction is strongly inhibited by inhibitors of glycolysis and glucose metabolism, but not by most mitochondrial inhibitors (408). Previous work suggests that electrons from glycolytic processes are passed through the PMRS by a poorly understood mechanism and can reduce WST-1 outside the cell. Some tetrazolium dyes, such as XTT, act very similarly to WST-1 (403), whereas other dyes, such as MTT, have completely separate intracellular mechanisms based on the different location of reduction. Finally, other extracellular dyes, such as ferricyanide, methylene blue (MB) and toluidine blue (TBO), differ from both WST-1 and MTT in their properties. Thiazine dyes (MB and TBO) are often reduced outside the cell and then taken up (404), and ferricyanide is reduced by the PMRS by a mechanism distinct from that of WST-1 (415).

Studies of reducible dyes illustrate that one must be aware of the type of dye used and the mechanism of reduction in order to interpret the results of specific experimental protocols. In many circumstances, tetrazolium dyes can be used to measure cellular growth and viability, but care needs to be taken when changes in cellular metabolism or PMRS activity could be occurring independent of changes in cell viability. This is especially important because the lack of knowledge of the specific PMRS mechanisms makes if difficult to know how a treatment may affect the reduction of an exogenous dye. Therefore, these dyes should (and will throughout this thesis) be used with the caveat that proper controls and an understanding of their reduction mechanism is required.

Goals of thesis

As detailed above, previous work has shown that the differences in longevity are often accompanied by differences in cellular and organismal stress resistance. Work in our lab showed that cells cultured from the long-lived Snell dwarf mouse were resistant to a variety of toxic stresses *in vitro*. Little is known of the pathways by which the stress resistance of cells from long-lived mice is established in vivo and then maintained during the course of in vitro passage. Furthermore, the lack of success of interventionist treatments attempting to increase lifespan through antioxidant drugs or antioxidant enzymes suggests that stress resistance may not be as simple as increasing an antioxidant or a detoxification enzyme. Pharmacological or genetic manipulations that augment antioxidant defenses often fail to prevent oxidative damage, but even when oxidation damage is clearly diminished, the interventions of this kind typical fail to increase longevity. In our cell culture model, we have a relatively simple (one cell type), readily available, and easy to use system for delineating the mechanisms of Snell dwarf mouse cellular stress resistance. The goal of my thesis is to move closer to finding a mechanism for the stress resistance of cultured cells from Snell dwarf mice.

Published studies, many of them detailed above, have suggested that changes in growth and metabolic signaling, such as those involved with calorie restriction and changes in the insulin/IGF-1 signaling, and were often accompanied by changes in stress resistance and changes in longevity. Therefore, the initial goal of my thesis was to examine the relationship between metabolism and stress resistance of the dwarf cells, and to find how differences in metabolism and stress resistance may be related. Many of the hypotheses tested during the dissertation project proved incorrect; pathways hypothesized to distinguish dwarf from normal cells and to contribute to stress resistance of the dwarf cells proved to function similarly in fibroblasts from control and mutant mice. Studies on Nrf2 and the PMRS proved more productive and the final chapter of my dissertation outlines these findings and suggests mechanisms that can tie together many of the previously unexplained findings in this cellular system.

On a larger scale, the goal of this work is to examine how specific pathways affect the aging process, in hopes of eventually finding ways to modulate the process in humans. Although duplication of the Snell dwarf phenotype in humans would have undesirable side effects, the mechanisms by which dwarf mouse aging is delayed are likely to be conserved in mammals. Thus learning more about the mechanism of cellular stress resistance is an early step in understanding organismal stress resistance and eventually testing to see whether artificially increasing stress resistance, with fewer side-effects, is both possible and sufficient to increasing longevity. This line of work, once completed, will be able to more thoroughly test the oxidative theory and may provide a means to manipulate the lifespan, or at the very least, the healthspan, of multiple types of animals, including humans.

Structure of thesis

The work is divided into 6 chapters, all of them focusing on a different part of the overall project. This chapter is devoted to introducing aging research and

providing a rationale and relevant background for the experimental approaches detailed throughout the thesis. Chapter II consists of much of my initial data on metabolic differences in dwarf cells, which became the basis for the project. Chapter III contains work related to the cellular metabolism of dwarf and control fibroblasts, most of which is classified as "negative data", but is informative in ruling out several pathways for future work. Chapter IV contains studies of stress resistance and PMRS function in cells from multiple mouse mutants and multiple species of animals, pointing to the relevance of this work towards the aging process in general. Chapter V includes work on differences between dwarf and control mice in Nrf2 and PMRS function. Lastly, Chapter VI summarizes and interprets the main conclusions of my dissertation work, and presents ideas about future directions. Some or all the work in each chapter has been included in manuscripts for publication, and the relation of these experiments to publications will be described at the beginning of each chapter.

Table 1.1. List of important antioxidants and their known role.

Antioxidant Type	Role
Methionine sulfide reductase	Reduces methionine sulfide protein adducts and prevents their infliction of further damage.
Superoxide Dismutase	Superoxide scavenger. Converts superoxide to hydrogen peroxide and oxygen.
Catalase	Hydrogen Peroxide scavenger. Converts H2O2 to water and oxygen.
Glutathione peroxidase	reduces lipid peroxides to alcohols and hydrogen peroxide to water.
Metallothionein	Scavenges metals and hydroxyl radicals.
Peroxiredoxin	Scavenges peroxide.
Thioredoxin	Reduces other antioxidants, detoxify ROS and refold proteins.
ascorbate	Cytosolic and extracellular antioxidant and electron donor.
α-tocopherol	Lipophilic (membrane) antioxidant and electron donor.
Coenzyme Q	Lipophilic (membrane) antioxidant and electron donor, also involved in respiration.
Bilirubin	Serum antioxidant.
Albumin	Serum antioxidant.

Table 1.2. Important antioxidant response element genes

Antioxidant Type	Role
Heme-oxygenase-1	Cleaves heme into biliverdin. May play a role in iron sequestering and other oxidative defense.
GCL (both subunite)	Catalyzes the rate-limiting step in glutathione synthesis.
Glutathione-S-transferase	Catalyzes the glutathionylation of damaged proteins and molecules.
Glutathione peroxidase	Reduces lipid peroxides to alcohols and hydrogen peroxide to water.
Metallothionein	Scavenges metals and hydroxyl radicals.
Peroxiredoxin	Scavenges peroxide.
Thioredoxin	Reduces other antioxidants, detoxifies ROS and refolds proteins.
Thioredoxin reductase	Reduced thioredoxin and maintains its activity. Also reduced lipid hydroperoxides.
NQO1	Donates electrons to reduce coenzyme Q, tocopherol in plasma membrane.
Malic Enzyme	Important in NADPH synthesis.
Catalase	Hydrogen Peroxide scavenger. Converts H_2O_2 to water and oxygen.

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CHAPTER II

CORRELATED RESITANCE TO GLUCOSE DEPRIVATION
AND CYTOTOXIC AGENTS IN FIBROBLASTS FROM
LONG-LIVED PITUITARY DWARF MICE

Foreword

Much of the work reported in this dissertation was stimulated in large part by previous work performed by Shin Murakami and Adam Salmon in the lab, finding that dermal fibroblasts isolated from the tails of young adult Snell dwarf mice were resistant to a variety of cytotoxic stresses. Drs. Murakami and Salmon focused their efforts on fibroblasts on the assumption that physiological changes in dwarf mice might be reflected in the properties of this easily cultured cell type, and might give insights into the factors that retard the development of signs and diseases of aging in young adult and middle-aged mice. My initial hypothesis was that glucose metabolism may be a determining factor in the survival of cells, and more specifically, may contribute to the increased cytotoxic stress resistance of cells from Snell dwarf mice. My hypothesis was derived from a part of previous work in our lab, specifically the requirement of a serum deprivation step previous to the addition of toxic stress. This serum deprivation step was necessary to observe the robust differences between dwarf and control cells. Separate work, published by Craig Thompson's lab and reviewed in Chapter I, led me to hypothesize that metabolic responses to growth in low serum might be important for differences between dwarf and control cells. Thompson's data showed that important intracellular factors including mTOR, Akt, hexokinase, Bax, and Bad were linked to growth factors through pathways regulating glucose metabolism and cell survival. This data inspired my initial work attempting to

examine the survival of dwarf and control cells when faced with low glucose in culture medium. While I found differences between dwarf and control cells in low glucose, I eventually discovered that I was not measuring survival, rather an activity (WST-1 reduction) linked in some way to glucose metabolism. Finding the basis of this activity, how it is linked to glucose metabolism, and why low glucose affects dwarf cells less so than control cells became the basis for the rest of this thesis. The initial observations of this work, published as Leiser SF*, Salmon AB*, and Miller RA. 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 makes up the whole of chapter II. It should be noted that this work was performed with Dr. Salmon, and his stress resistance data makes up much of the correlation tables and correlation figures shown (Figures 2.3 and 2.4). I also had generated cellular stress resistance data in agreement with Dr. Salmon's, but his data set was more extensive and therefore was used in the publication. The writing of the manuscript was split evenly between Dr. Salmon and myself, with Dr. Miller providing extensive editorial input. Thus, Dr. Salmon and I were listed as coauthors on this work, as we each provided approximately half of the data and writing that became the published manuscript.

Abstract

Fibroblast cell lines derived from the skin of young adult mice of the long-lived Snell dwarf mutant mouse stock have been shown to be resistant to the cytotoxic effects of multiple agents, including hydrogen peroxide, cadmium, heat, ultraviolet light, and the carcinogen methyl methanesulfonate. Snell dwarf fibroblasts are here reported to differ from control cell lines in two other respects: they are relatively resistant to the metabolic inhibition induced by low glucose concentrations, and also resistant to the effects of the mitochondrial poison rotenone, a blocker of Complex I of the electron transport chain. Furthermore, analysis of cell lines derived from a group of genetically heterogeneous mice established that cell lines resistant to peroxide-induced cytotoxicity were also relatively resistant to death induced by paraquat, cadmium, and ultraviolet light. 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

The Pit1^{dw} mutation (Snell dwarf mutation, *dw/dw*) 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;2). Like Ames dwarf mice (Prop1$^{df/df}$),

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with their similar set of hormonal abnormalities (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 (4;5). The lifespan extension of the Snell dwarf mutant seems to represent a retardation of multiple forms of late-life pathology, including joint disease (6), immune and connective tissue changes (4), and changes of the kidney glomerulus and eye lens (5). Similarly, Ames dwarf mice show retardation in age-related cognitive failure (7) and neoplasia (8). It seems likely that the anti-aging effects in the Ames and Snell 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 (9), as well as in mice with low IGF-I receptor levels (10) and in mice with absence of the growth hormone releasing hormone (lit/lit mice, mutant for Ghrhr) (4).

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 (11;12). 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 (11), 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 (11).

Motivated by published evidence that abnormalities of glucose metabolism could influence apoptotic cell death (13-15), we have now evaluated differences between Snell dwarf and control fibroblast cell lines in their metabolic responses to glucose deficits and to a mitochondrial poison that blocks an early step in the electron transport chain. The results show that cells from Snell dwarf mice are not only resistant to lethal agents, but also to non-lethal agents that interfere with cellular energy metabolism. Furthermore, those cell lines from genetically heterogeneous (normal) mice that are most resistant to lethal injury tend also to be resistant to metabolic inhibition, suggesting that the two forms of stress resistance share common elements.

Methods

Animals. 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.

Primary and secondary cell cultures. Distal tail skin biopsies approximately 3-5 mm in length were obtained from 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-

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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 2.5×10^5 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 passage 0 cells) were either (1) split six-fold or nine-fold by volume to create first passage cells, with twofold or threefold dilutions at each subsequent passage or (2) split and seeded at a density of 1×10^5 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 water, 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 second passage (6 days after seeding), confluent cells were used for assessment of stress resistance.

Assessment of fibroblast resistance to lethal stress. Six days after seeding, second 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 approximately 18-hour overnight incubation, cells were washed once with warm 1X PBS and incubated in 37° DMEM supplemented with 2% bovine serum albumin (BSA, Sigma), antibiotics and fungizone for approximately 24 hours. Cells were then exposed to a range of doses of one of the cytotoxic stressors. For UV light testing, cells were washed with warm 1X PBS, then irradiated with doses of UV light (254 nm at 5.625 J/m2/s) in 100 µl of 37° Dulbecco's PBS (Biowhittaker-Cambrex Life Sciences). Cells were then incubated in 37° DMEM supplemented with 2% BSA, antibiotics and fungizone, and their survival was measured 18 hours later by a test based on reductive 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 37° DMEM. Cells were then washed with warm 1X PBS and incubated in 37° 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.

Assessment of cell metabolism in low glucose medium. Cells harvested and grown to the second passage as above were plated at a density of 3 x 10^5/ml in DMEM with 20% fetal bovine serum with antibiotics and fungizone. 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). 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. After a one-hour incubation in these media, WST-1 was added and formazan conversion measured. In some experiments cells were tested to see if low glucose culture had led to cell death; in this instance cells were returned to high-glucose DMEM and measured again by WST-1 and, separately, tested for continual thymidine uptake by scintillation counting as previously described (12;16;17).

Inhibition of cell metabolism by rotenone. Cells harvested and grown to the second passage as above were plated at a density of 3 x 10^5/ml in DMEM with 20% fetal bovine serum with antibiotics and fungizone. 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.

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

Fibroblast cell lines from long-lived mutant Snell dwarf mice are relatively resistant to the metabolic effects of glucose deprivation. In previous work, our laboratory has noted that fibroblast cell lines from Snell dwarf mice are resistant to death induced by a wide range of toxic agents (11;12;17), including oxidizing agents, heavy metals, heat, ultraviolet light, and DNA alkylating agents. Because of evidence suggesting that altered intracellular glucose levels could regulate susceptibility to apoptosis (13;14), we evaluated the effects of diminished glucose on conversion of the tetrazolium salt WST-1 to its colored formazan product, a reaction that can be catalyzed by mitochondrial redox enzymes (18) as well as by non-mitochondrial enzymes in some cell types (18;19). As shown in Figure 2.1 (left), cells incubated for 60 min at glucose concentrations below 0.4 mg/ml show dose-dependent decline in WST-1 reduction. Additional experiments, not shown, demonstrated that the change in WST-1 cleavage was detectable as early as 20 minutes after the switch to low glucose medium. In spite of this change in metabolic function, the cells appeared normal and healthy under the microscope in the low glucose concentrations. The cells remained fully viable even 48 hr after initiation of glucose deprivation, with full recovery of WST-1 metabolic activity 1 hr after return to regular growth medium with glucose at 1 mg/ml. In addition, we found that glucose withdrawal for a period of 1 hour or 24 hours had no effect on the levels of DNA synthesis in either *dw/dw* or control cells as measured by radiolabelled thymidine incorporation (not shown). Although WST-1 is often used in tests for cell viability, it is important to note that the decline in WST-1 reduction under low glucose conditions is not accompanied by cell death. The lower level of WST-1 reduction seen in low glucose conditions seems likely to reflect alterations in metabolic

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pathways needed to produce the reducing equivalents for WST-1 cleavage at the plasma membrane (18-20).

The experiment shown in Figure 2.1 (left) suggests that fibroblasts from young adult Snell dwarf donors are resistant to this inhibitory effect of low-glucose medium, and retain relatively high levels of WST-1 metabolic activity, compared to cells from control mice, across a wide range of glucose concentrations. To test the reproducibility of this result, we conducted tests of glucose-mediated inhibition in nine experiments involving cell lines from 23 dwarf and 20 littermate control mice. The results are shown in Figure 2.1 (right panel). Although there is a good deal of overlap between the two groups, there is a clear and statistically significant ($p < 0.001$) tendency for cell lines from dwarf mice to be relatively resistant to the effects of low glucose.

Dwarf fibroblasts are also relatively resistant to the mitochondrial poison rotenone. Reductive cleavage of WST-1 is thought to depend on availability of NADH or NADPH (18), whose concentration depends in part on mitochondrial activity. We therefore evaluated the hypothesis that dwarf and control fibroblasts might differ in their resistance to the effects of rotenone, which inhibits Complex I of the mitochondrial electron transport chain. Furthermore, the effects of rotenone on WST-1 signal inhibition are fully reversible at the doses used. For example, when cells are exposed to 5 µM rotenone for 4 hr and then allowed to recover in rotenone-free medium overnight, cell viability as measured by WST-1 reduction remains greater than 80% of untreated cells.

Figure 2.2 (left) shows a typical experiment comparing dwarf-derived to normal-derived cells. WST-1 reduction is inhibited by rotenone in both control and dwarf cell lines, but the concentrations of rotenone needed to produce 50% inhibition are higher in dwarf-derived cell lines than in control cells. In tests of 6 pairs of dwarf and control cell lines (Figure 2.2, right panel), we found that the mean inhibitory dose for dwarf cells (3.2 µM) was more than twice that for control cells

(1.5 µM; p < 0.02).   Thus cells from adult Snell dwarf mice are resistant to metabolic inhibition caused either by low glucose or by inhibition of mitochondrial Complex I.

Correlated resistance to multiple forms of cell injury and inhibition.   Our previous work (11;12;17) 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 2.1 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 (11). Scatterplots of these relationships are included in the top panels of Figure 2.3, 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 2.1. With one exception (responses of littermate controls to UV), each of these correlations is also positive with R ≥ 0.3, and four of the correlations reach statistical significance. The responses of the Snell dwarf mice are also included in Figure 2.3 (middle row). Table 2.1 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 2.3 (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.

Some of these cell lines, along with others from a separate pool of donors, were also evaluated for glucose sensitivity as well as for resistance to multiple forms of cytotoxic stress. Table 2.2 shows the results of a correlation analysis analogous to that shown in Table 2.1. 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 2.4 shows illustrative scatter plots. Results for the littermate controls for the Snell dwarf mice, also shown in Table 2.2 (third line) were far less

dramatic, and only reached significance for the association between glucose withdrawal and paraquat resistance ($p < 0.03$).

Discussion

Fibroblasts derived from tail skin of adult Snell dwarf mice differ from control fibroblasts in multiple ways. In serum-free conditions, they are resistant to death induced by oxidative agents (hydrogen peroxide and paraquat), heavy metals (cadmium), heat, ultraviolet light, and DNA damaging agents (methyl methanesulfonate) (11;12;17). In the work reported here, we have shown that these cells are also relatively resistant to two conditions that inhibit the reductive cleavage of the tetrazolium salt WST-1, i.e. the mitochondrial poison rotenone, and medium containing unusually low glucose levels. We do not yet have a good understanding of the metabolic changes that underlie any of these forms of resistance, but hope that further exploration of resistance to cytotoxic stresses, glucose withdrawal, and rotenone may lead to a coherent model the accounts for each of these unusual properties.

The correlation data presented in Tables 2.1 and 2.2 and in Figures 2.3 and 2.4 suggest strongly that these properties may be regulated by an overlapping set of cellular mechanisms. For cell lines derived from UM-HET3 mice (21), 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 (22;23), 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

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that reduce the effects of oxidative damage (11). Thus the correlation between resistance to peroxide and resistance to UV irradiation cannot easily be attributed to anti-oxidant 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 over-expression 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 2.2, Figure 2.4). 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 (22;23). 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.

Further analysis of the specific links between glucose withdrawal and resistance to cytotoxic stress will depend, in part, on an improved understanding of the basis for differences in cellular reduction of the tetrazolium salt, WST-1, on which our rotenone and glucose assay data are based. This agent is often used in assays for cell number and cell viability (24-26), because reduction of WST-1 to its formazan product is proportional to viable cell number under circumstances where all live cells are equally active. Little is known, however, of the nature and subcellular location of the enzyme or enzyme systems that are responsible for reductive cleavage of WST-1 in fibroblasts. Although it is commonly asserted that the reduction of WST-1 requires electrons donated by NADH or NADPH, a study of WST-1 reduction by a variety of cell lines has suggested the reaction is conducted largely by extracellular enzymes, probably at the plasma membrane (18;20;27). Furthermore, reduction of WST-1 is enhanced, rather than inhibited, in cell lines that lack mitochondria altogether (19). In addition, we found no effect of glucose withdrawal on reduction of a second tetrazolium salt, MTT, whose reduction is thought to represent activity of mitochondrial and other intracellular enzymes, rather than the extracellular reduction of WST-1 (27). We see no differences between dwarf and normal cells in levels of MTT reduction at any glucose level tested (Leiser, unpublished). Taken together, these results suggest that further study of the control of the plasma membrane enzymes that reduce WST-1 is likely to help elucidate the basis for resistance to glucose withdrawal seen in cells from dwarf mice.

A recent paper (28) has shown that WST-1 reduction is mediated, at least in part, by a member of the ECTO-NOX family of NADH oxidases, whose members are thought to communicate information about surface changes in oxidative status to other, nearby cells (29). A second element of the same protein complex is the protein gp96, a glucose-sensitive member of the hsp-90 family of heat shock

proteins (28). Further work will be needed to learn whether differences in these extracellular proteins contribute to differences between dwarf and control cells in sensitivity to glucose levels, and, by extension, also influence resistance to the cytotoxic injury caused by cadmium and peroxide.

The rotenone resistance exhibited by cell lines from dwarf mice points towards pathways that are connected to Complex I of the mitochondrial electron transport chain. Although at higher doses rotenone leads to cell death, the diminution of WST-1 reduction at the dose range we have used in this study is fully reversible for at least 4 hours, and cells return promptly to control levels of WST-1 reduction when the toxic agent is removed. In addition, we find that rotenone, like glucose deprivation, does not alter the rate of metabolism of MTT across the time and dose ranges used in our study. Thus it is currently unclear how rotenone-mediated inhibition of Complex I produces a diminution of WST-1 reduction in these fibroblasts. Complex I of the electron transport chain uses electrons transferred from NADH, and it is possible that differences between normal and dwarf cells in sensitivity to altered NADH or NADPH concentrations might contribute both to rotenone resistance and altered responses to low glucose concentration.

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 (30;31), and the activity of pro-apoptotic factors (15;30;32). Glyceraldehyde-3-phosphate dehydrogenase, similarly, has been implicated in the control of DNA repair and apoptosis (33;34). 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 (14). More generally, glucose signals modulate the activity of Akt and its downstream agonist mTOR, both of which help to regulate cell survival (13). Further, in

human colon carcinoma cell lines, Akt protects from oxidative- and glucose deprivation-induced death through FOXO3a (35). Akt, JNK1, and ERK all have been shown to be involved in various models of resistance both to cell death and glucose deprivation (35-39). Similarly, both glucose withdrawal and oxidative stress have been shown to increase a number of the same proteins including CHOP and GRP-78 (40;41), mediated in part by the actions of PKCδ (42;42-45) 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 (46). 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.

Table 2.1.

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)**

Table 2.1. Correlation of resistance to peroxide induced cell death to paraquat, cadmium, or UV light induced cell death for fibroblast lines from four mouse strains

*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 2.2

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)

Table 2.2. Correlation of resistance to glucose withdrawal to peroxide, paraquat, cadmium or UV light induced cell death for fibroblast lines from three mouse strains

*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.
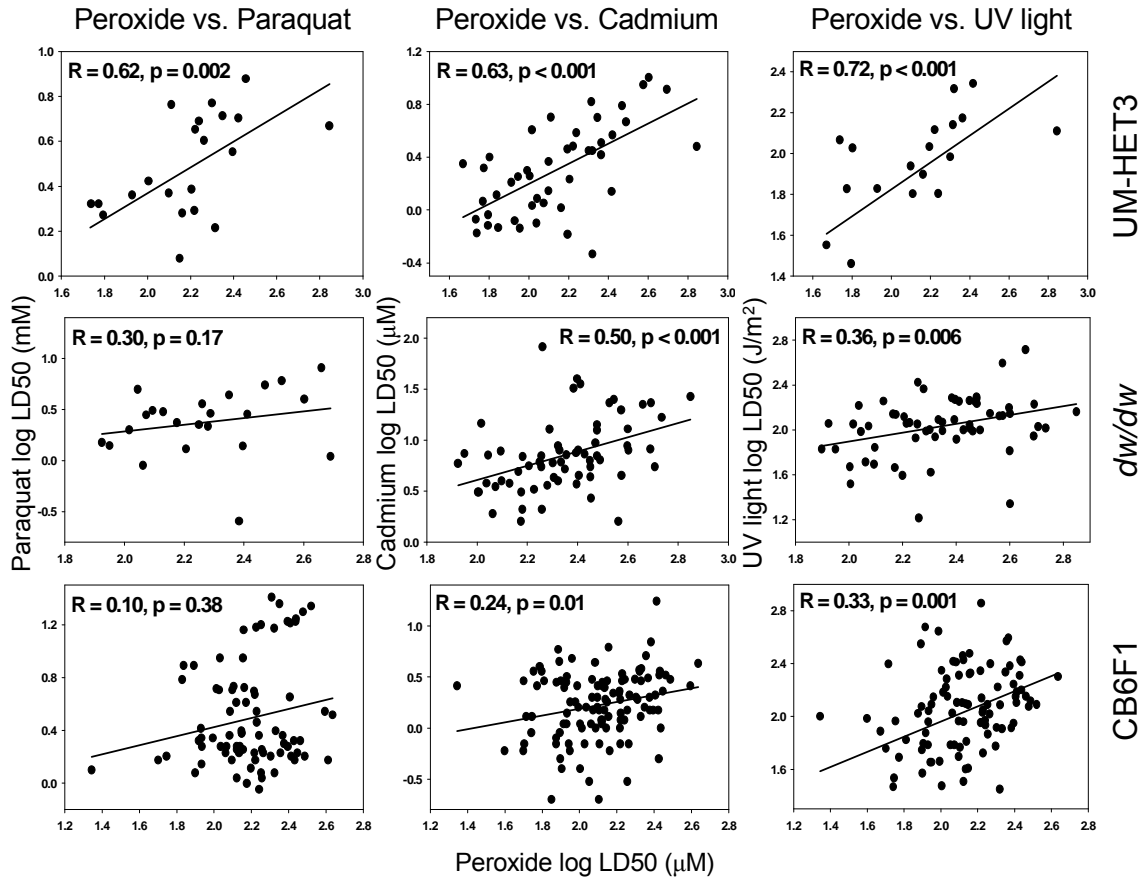
Figure 2.1.
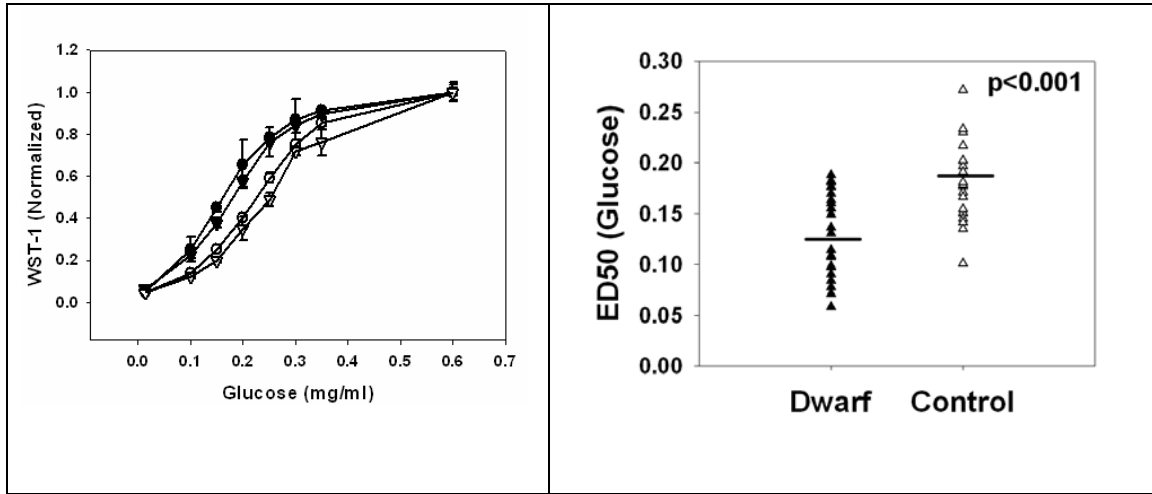
Figure 2.1. Differential sensitivity of dwarf and control fibroblasts to culture in low glucose medium. The left panel shows a typical experiment, involving cell lines from two separate dwarf donors (filled symbols) and from two separate littermate controls (open symbols). The Y-axis shows the WST-1 signal, normalized to the value of 1.0 for cells grown in complete medium with 4 mg/ml glucose. Error bars represent the standard error among triplicate wells assayed at each glucose concentration. There were no significant differences in the level of WST-1 signal between dwarf and control cultures in complete medium with normal, high levels of glucose (not shown). The right panel summarizes the entire series of glucose experiments; each point represents a cell line from a different dwarf or control donor. ED50 is the concentration of glucose at which WST-1 signal was 50% of the level for the same cell line in complete medium with a normal, high level of glucose. Horizontal lines show means for each group of cultures.
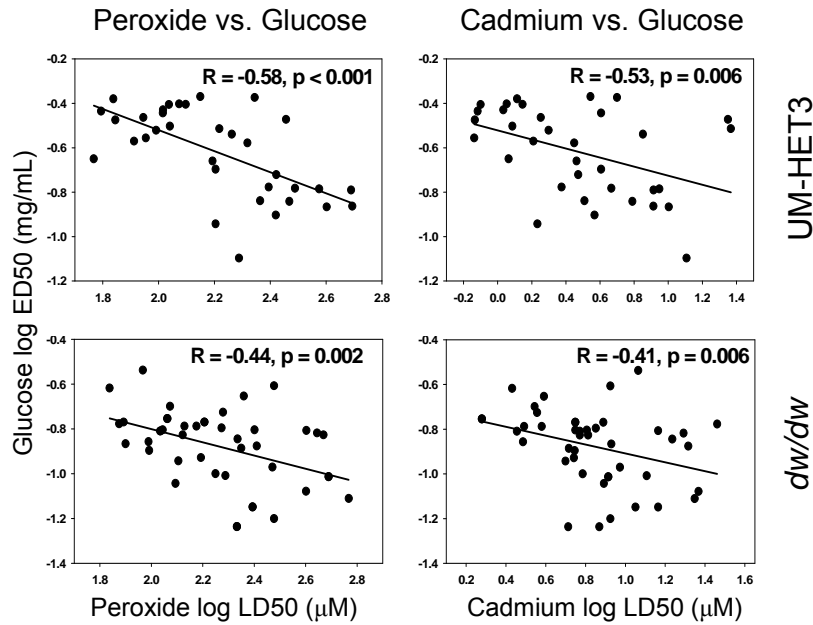
Figure 2.2.
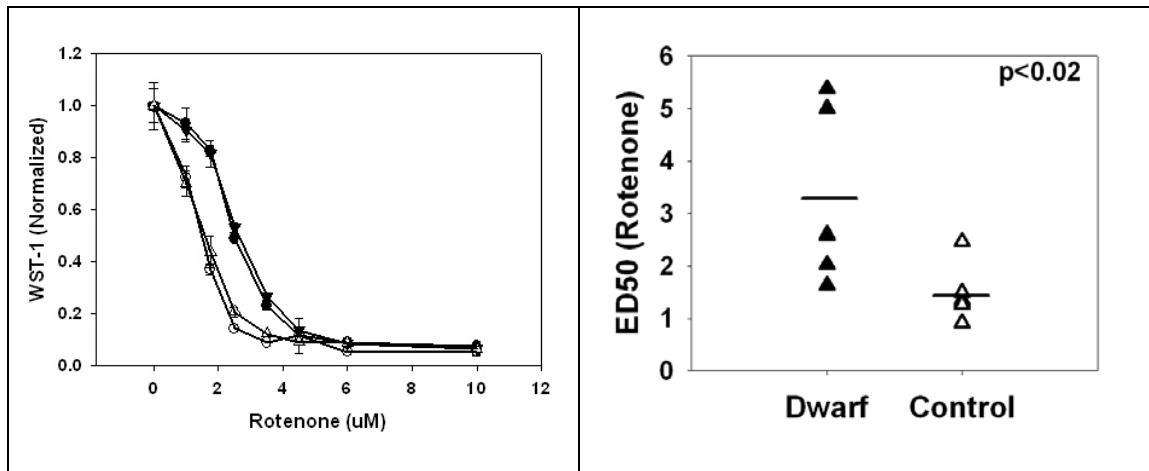
Figure 2.2.   Differential sensitivity of dwarf and control fibroblasts to culture in varying doses of the mitochondrial inhibitor rotenone. The left panel shows a typical experiment, involving cell lines from two separate dwarf donors (filled symbols) and from two separate littermate controls (open symbols). The Y-axis shows the WST-1 signal, normalized to the value of 1.0 for cells in the absence of rotenone. Error bars represent the standard error among triplicate wells assayed at each concentration of rotenone. There were no significant differences in the level of WST-1 signal between dwarf and control cultures in the absence of rotenone (not shown). The right panel summarizes the entire series of rotenone experiments; each point represents a cell line from a different dwarf or control donor. ED50 is the concentration of rotenone at which WST-1 signal was 50% of the level for the same cell line in complete medium. Horizontal lines show means for each group of cultures.

Figure 2.3.

Figure 2.3. **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/m2). 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 2.4.

Figure 2.4. 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 log of the concentration of glucose (in mg/ml) that leads to a 50% reduction in WST-1 conversion (ED50). Each symbol shows LD50 and ED50 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.

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CHAPTER III

METABOLIC STUDIES IN CELLS FROM LONG-LIVED
DWARF MICE: MITOCHONDRIA, GLUCOSE METABOLISM
AND DYE REDUCTION

Foreword:

The data for this chapter involve metabolic resistance work, most of which was performed shortly after the initial findings described in chapter II. Having shown that glucose deprivation and rotenone exposure were less inhibitory to dwarf cells than control cells, I attempted to find broad-based metabolic differences in the cell types to explain the differences I had observed. In the end, much of this work would be considered negative data, but was valuable because it ruled out several areas of study and also gave further insight into the phenomena observed in chapter II. A small portion of this data (Figure 3.4), was integrated into an accepted manuscript, Mechanisms of Stress Resistance in Snell Dwarf Mouse Fibroblasts: Enhanced Antioxidant and DNA Base Excision Repair Capacity, But No Differences in Mitochondrial Metabolism. Page MM, Salmon AB, Leiser SF, Robb EL, Brown MF, Miller RA, Stuart JA. 2009 Free Rad. Biol. Med. (Accepted). The rest of this work remains unpublished, mostly because it does not show a mechanism for previous observations.

Abstract

The impact of cellular metabolism on growth and survival is well documented. Data here show that despite previous work showing that isolated primary dermal fibroblasts from long-lived Snell dwarf mice resist the metabolic inhibition of

WST-1 reduction by culture in low glucose or exposure to rotenone, dwarf and control cells are similar to one another in many aspects of glucose metabolism and gross mitochondrial content. While these data serve largely to disprove hypotheses about increased metabolism in dwarf cells, we also show that the initial rotenone data is repeatable, and there are other small differences between dwarf and controls cells in glucose uptake and resistance to mitochondrial toxins. Also, this work shows that inhibition of WST-1 reduction occurs quickly after exposure to rotenone or low glucose media and does not kill the cells, and that reduction of WST-1 is inhibited by glycolytic inhibitors and acts similarly to other extracellularly reduced dyes.

Introduction

While the use of exogenous dyes to measure cellular growth and viability is widely accepted, the molecular mechanisms for cellular reduction of dyes are often unknown or misunderstood (1;2). Our previous work, utilizing primary dermal fibroblasts from the long-lived Snell dwarf mouse, showed that these dwarf cells were resistant to the metabolic effects of low glucose medium and rotenone exposure as measured through an extracellular dye, WST-1 (3), that is often used in assays cellular growth and viability (Roche). Since previous work showed that reduction of WST-1 by cultured primary fibroblasts can be inhibited without causing cell death, we reasoned that differences in fuel utilization of related aspects of cellular metabolism might regulate the level of WST-1 reduction. In order to test how cellular metabolism might cause differences in WST-1 reduction between dwarf and normal cells, we focused on the known effects of low glucose and rotenone exposure. More specifically, we examined the main pathways of cellular metabolism, glycolysis and mitochondrial cellular respiration, and also tested the properties of several exogenous dyes, including WST-1.

Fibroblasts are known to rely mostly on glycolytic metabolism, especially when cultured in high glucose environments (4;5). The basic cellular metabolism in fibroblasts involves glucose uptake through specialized transporters (Gluts), metabolism of glucose to pyruvate through glycolysis, and then conversion of pyruvate to lactate to recycle NAD before releasing the lactate into the media. Thus, diminution of extracellular glucose levels leads to declines in glucose uptake, glycolysis, and cellular metabolism. At doses that inhibit the electron transport chain, rotenone would be expected to cause an increased rate of glycolysis in response to loss of mitochondrial ATP production, especially in short term assays (6;7). Since our assays are short-term exposures (~4 hours) to rotenone, it is likely that glycolysis rates would be increased by rotenone at the doses and exposure times used.

A decrease in glucose or the addition of rotenone in culture medium will also affect the metabolism of mitochondria. In short term assays, low glucose in the medium lowers intracellular glucose uptake and glycolysis rates in cells, leaving less pyruvate available for the mitochondrial citric acid cycle and subsequent mitochondrial ATP production. Rotenone, at sufficiently high doses, affects the mitochondria directly, by binding to electron transport chain complex I and preventing the passing of electrons from NADH to coenzyme Q, largely disabling the electron transport chain (6;8). Since disruption of mitochondria can lead to changes in metabolism and apoptosis, there is an expectation that low glucose or rotenone exposure could have wide-ranging affects on cells (9;10). As a working hypothesis, I asked whether these effects could explain the correlation between glucose withdrawal and stress resistance observed, through effects on mitochondria.

Metabolism, both glycolytic and mitochondrial, has been implicated in respect to aging and cellular stress resistance. The first used and most studied way to delay aging is through calorie restriction (CR) (11). CR is known to have effects on a variety of metabolic pathways, including mitochondrial electron transport,

metabolite availability, and energy efficiency (12;13). Mitochondria have been implicated in aging through several pathways (discussed in chapter I), including apoptotic decisions (10), production of reactive oxygen species (ROS)(14), and maintenance of cellular energy levels (15). Chapters I-II also document how changes of cellular metabolism in response to serum withdrawal can affect cellular metabolism and commitment to apoptosis (16;17). This, and the data in chapter II showing a correlation between metabolic resistance and stress resistance in cultured cells, suggests that cellular metabolism and stress resistance may be related, and when considered in respect to other data (18;19), may relate to aging. Thus, we examined if differences in glycolytic or mitochondrial pathways in dwarf cells could be responsible for the resistance to lethal and non-lethal stresses.

Methods

Animals. See Chapter II.

Primary and secondary cell cultures. See Chapter II.

Low Glucose/Metabolite Assays. See Chapter II

Inhibitory Assays. See Chapter II

Mitochondrial Measurements. The mitochondrial content and membrane potential of fibroblasts from Snell dwarf and normal littermate controls were measured by flow cytometry using MitoTracker^{TM} probes. Briefly, confluent cells grown at 20% O_2 were placed in complete or serum-free media for 24 hours, after which the cells were washed thoroughly in DMEM media, without serum, immediately prior to their incubation with MitoTracker^{TM} Green (100nM) and MitoTracker^{TM} Red (250nM) probes for 30 min. The cells were then washed twice with ice-cold PBS before being scraped into test tubes. The cells were

analyzed on a Beckman-Dickson FACSCalibur with excitation at 488nm and emission at 530nm for MitoTracker[TM] Green and 585nm for MitoTracker[TM] Red and gated for single cells.

In addition, activities of specific complexes of the electron transport chain were measured by the lab of Dr. Placido Navas, using previously published techniques (20), using cell lines provided to them from our laboratory.

Inhibitor Survival. Cells were plated confluently ($3X10^6$ cells per well) in 96-well plates and allowed to adhere overnight. Media was removed, cells were washed with PBS, media (DMEM, 20% FCS, pen/strep, fungizone) containing varying concentrations of inhibitors were added, and cells were incubated for 1 hour at 37° and 5% CO2 before addition of 5 μl of WST-1 (Roche). After 3 hour incubations with WST-1, plates were read at 585nm on a 96-well spectrophotometer to obtain inhibition results. After reading, inhibitory media was removed, cells were washed in PBS, and returned to standard media. After a 24 hour recovery period cells were tested again with WST-1 to measure survival. Results were verified by measuring thimidine uptake as described previously (21).

Glucose Uptake. Cells were plated confluently on 60 mm plates overnight before medium was removed and cells were washed twice with warm Krebs Ringer phosphate (KRP) buffer ((mM) NaCl 154, KCl 5.6, $MgSO_4$ 1.1, $CaCl_2$ 2.2, NaH_2PO_4 0.85, Na_2HPO_4 2.15), and incubated in KRP for 30 minutes. Subsequently, 2 ml of mixed H^3 2-DOG and 2-DOG (1 mM) in KRP was added to the cells for 5 minutes, before being removed and quenched with phloretin (0.2 mM). Cells were subsequently lysed in 0.1% SDS and scraped; after which ½ of the lysate was used for protein content and ½ was measured for H^3 using scintillation counting (22).

Protein content measurement. Protein content was measured by DC protein assay (BioRad), as described by the manufacturer.

Enzymatic Activity Assays. Whole cell lysates were prepared from confluent cells plated overnight on 100 mm dishes. Cells were washed with ice-cold PBS, scraped into a tube and centrifuged at 1000 x g. PBS was removed and 100 μl KPB buffer (34 mM KH2 PO4, 16 mM K2HPO4, (pH 7.0), 1% Triton X-100, and protease inhibitor cocktail (Fisher) was added. Cells were incubated for 15 minutes, nuclei were spun down, and supernatant was flash frozen in liquid nitrogen and stored at -80° until use. Assays were performed on cell lysates (20 μg) using kits measuring the increase in NADH from GAPDH (Ambion, Austin, TX) or LDH (Roche) activity, as described by the manufacturer.

Immunoblots. Lysates for western blots were prepared in RIPA buffer. Lysates were aliquotted and stored at -80° until the date of use. All blots were run on SDS-PAGE gels containing 10% acrylamide (BioRad). All primary and secondary antibodies for Nrf2, Nrf1 and Keap1 were obtained from Santa Cruz Biotechnology. Secondary antibodies conjugated to alkaline phosphatase were then added, and blots were read after enhanced chemifluorescence (ECF, Amersham) addition on a Storm 840 fluorescent scanner. Quantification was performed using ImageQuant software.

Metabolites. Cells for metabolic studies were plated confluently in 60mm dishes. Following overnight incubation, cells were washed three times in PBS and treated in parallel with various metabolic stresses for 2 hours. They were then washed in ice-cold PBS and lysed with either trichloroacetic acid (TCA) or sodium hydroxide (NaOH). Lysates were centrifuged to remove protein and neutralized to ~pH 6.5 before testing. Lysates were then separated and quantified on an HPLC column as previously described (23).

Statistical analysis. Statistical analyses were performed using paired t-tests (two tailed) for comparison between dwarf and control cells, because each dwarf and control cell line pair was tested together. Graphs show the mean of combined

experiments and error bars represent the standard error of the mean. Differences between treatments were calculated using repeated measures ANOVA. For calculation of LD50s, mean survival was calculated at each dose and the LD50 was calculated using probit analysis using NCSS software (NCSS, Kaysville, UT).

Reagents. Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, St. Louis, MO.

Results

Most mitochondrial inhibitors do not inhibit WST-1 reduction at non-lethal doses

Previous work showed that cells from dwarf mice are resistant to the inhibitory effects of rotenone, an inhibitor of mitochondrial complex I (3). Figure 3.1 shows additional data, repeating and expanding the original data set, and showing that dwarf cells are nearly two-fold more resistant to rotenone mediated inhibition of WST-1 reduction compared to cells from littermate controls. To see if other mitochondrial inhibitors acted similarly on our cells, we performed similar assays using other mitochondrial inhibitors (Figure 3.2), including amytal (complex I, B), piericidin (complex I, not shown) TTFA (complex II, A and C), antimycin A (complex III, D), and cyanide (complex IV, not shown). None of the other mitochondrial inhibitors (Figure 3.2A-B), i.e. none except for rotenone, were successful in inhibiting WST-1 reduction at concentrations that did not also lead to cell death. Dwarf cells did show small but significant (TTFA) or nearly significant (antimycin A) increases in resistance to the lethal effects of mitochondrial inhibitors. However, the effects of rotenone, i.e. inhibition of WST-1 signal without cell lethality, were not mimicked by any of the other tested mitochondrial inhibitors, including other complex I inhibitors.

Dwarf and control cells do not differ in gross mitochondrial properties

To test if dwarf cells have an increase in mitochondria or mitochondrial activity, we measured the content, membrane potential, and electron transport chain activity of dwarf and control fibroblasts. Figure 3.3 shows the relative amount of mitochondrial protein (MitoTracker Green), membrane potential (MitoTracker Red), and the ratio of membrane potential to protein in dwarf and control cells in control and serum free conditions. There were no significant differences between normal and dwarf cells, nor trends toward changes, in any of the measurements, nor differences in levels of citrate synthase activity, often used as a surrogate for mitochondrial content. Figure 3.4 shows that the lack of mitochondrial differences extend to the electron transport chain, where work from mitochondria isolated and measured by the lab of Dr. Placido Navas found no differences in any complex activity or inhibitory effect between dwarf and control cells. Thus, data suggest that dwarf and control fibroblasts are very similar in content and activity of mitochondria.

Dwarf cells have increased glucose uptake, but are similar to control cells in other steps in glycolysis

We next examined the cytosolic glucose metabolic pathway. First, we examined the cellular uptake of glucose, using a tritiated glucose analog (2-DOG), which can be taken up and phosphorylated but not hydrolyzed. Results (Figure 3.5) show that dwarf cells take up more glucose than control cells, with differences between 25% (without serum) to about 35% (with serum). These differences were statistically significant ($p < 0.03$), but were not accompanied by an increase in the expression of the constitutive glucose transporter, Glut1, or the insulin sensitive glucose transporter, Glut4.

We also examined the expression of other metabolically important proteins (Figure 3.6), including hexokinase I (A), glycogen synthase kinase-3 (B),

phosphofructokinase-1 (not shown), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)(not shown). As summarized in Figure 3.6, none of the proteins of interest differed to a significant extent between dwarf and control cells, and the activities of GAPDH (C) and lactate dehydrogenase (D) were also unchanged by genotype or condition.

We next examined the levels of important metabolites that could be modified by changes in cellular metabolism. Figures 3.7 and 3.8 show the levels of adenosine and guanine related metabolites in a variety of metabolically stressful conditions, including serum free medium, low glucose, and rotenone conditions. The results show very few differences between dwarf and control cells, with the exception of sporadic increases of control cells over dwarf cells in the levels of ADP, AMP, and GDP. However, there were no consistent trends across the conditions and little evidence to suggest that differences between dwarf and control cells in steady-state levels of these metabolites could contribute to the functional differences in resistance to stress.

NAD(P)(H) responds similarly to metabolic stress in cells from dwarf and control mice.

Because the enzymes responsible for WST-1 reduction require NAD(P)H, and because both glucose deprivation and rotenone exposure affect NAD(P)H levels, we also evaluated the redox status of NAD(P)H in dwarf and control fibroblasts under control and stressed conditions (Figure 3.9). As expected, NADH levels were reduced in low glucose media and increased by rotenone, but there were no significant differences between the dwarf and littermate controls in any of the conditions tested. Similarly, there were no differences between dwarf and control cells in levels of NADPH or in the ratio of NAD(P) to NAD(P)H in any condition. There were some small but statistically significant increases in normal NAD levels in several conditions, but no concomitant changes in NADH or NAD:NADH to suggest these changes might be physiologically important. These data

suggest that differences between dwarf and normal cells in the ability to reduce WST-1 are unlikely to be due to differences in levels of NAD(P) metabolites, but do not rule out the possibility that differences in specific cellular compartments (e.g. cytosolic) in NAD(P)H could be important for exogenous dye reduction by dwarf cells under metabolic stress (note: these data are included in chapter V as well, in abbreviated form, in the context of a paper submitted for publication).

We next examined the some of the attributes of the systems responsible for WST-1 reduction in our fibroblast system. As shown in Figure 3.10, the decline in WST-1 reduction in low glucose medium is non-lethal, and occurs quickly, with measurable effects just 30 minutes after culture in low glucose medium. Reduction of WST-1 can be increased by the addition of lactate, and is inhibited by pyruvate, by the glucose analog 2-DOG, and by the glycolysis inhibitor iodoacetate. Cell death was minimal for each of the experiments illustrated in Figure 3.10 (data not shown), suggesting that the effects of the metabolites lactate and pyruvate, which push NAD recycling toward or away from NADH, respectively, affect WST-1 reduction directly rather than through lethal effects on the fibroblasts. The work also shows that glucose metabolism and glycolysis are necessary for WST-1 reduction, suggesting that cytosolic NADH and/or other electron donors may be important for WST-1 reduction. Lastly, Figure 3.11 shows that a related tetrazolium dye, MTT, which unlike WST-1 can enter cells, differs from WST-1 in its functional properties, in that its reduction is unaltered either by low glucose medium or after rotenone exposure. This observation strongly suggests that the alterations in WST-1 status induced by rotenone or low glucose do not reflect changes in intracellular levels of reducing equivalents. In contrast, extracellular electron acceptors, such as toluidine blue O, methylene blue, and XTT (not shown), show similar inhibition of reduction, although not to the same extent as WST-1 in every case (see figure 3.11D). Together, these data show that reducing equivalents for WST-1 reduction are likely to come from glucose metabolism, and that WST-1 is likely to be reduced extracellularly by the PMRS, in agreement with previous work (1;2).

Discussion

The results suggest that changes in mitochondrial content or baseline activity, or glycolytic differences in dwarf cells, are each unlikely to be the cause of the previously observed metabolic and cytotoxic stress resistance of dwarf cells. Since mitochondrial content, metabolic enzymes, and metabolite levels were examined only at the level of the whole cell, we cannot rule out the idea that compartmentalized changes, such as alterations in membrane-bound or membrane associated enzymes or organelles, might be responsible for the functional properties of dwarf cells, but no work to date has implicated these pathways with any consistency. The data also suggest that some product of glycolysis, probably production of cytosolic NAD(P)H or other electron donors, can inhibit WST-1 reduction, since glycolytic inhibitors such as 2-DOG and iodoacetate inhibit WST-1 reduction.

The mitochondrial data in this chapter suggest that there are no differences in the content, membrane potential, or electron transport chain activity between dwarf and control cells. Repeated data using rotenone to inhibit WST-1 reduction proved consistent, solidifying previously work (Chapter II), but no other inhibitors, including complex I inhibitors, were able to inhibit WST-1 reduction without toxicity. There were changes in dwarf cellular resistance to toxicity from mitochondrial electron chain complex II and III inhibitors, but the differences were small and the mechanism of cell death seen when these toxins were used at lethal doses, is at this time unknown. We did not directly evaluate enzymes of the citric acid cycle, but metabolite data did not suggest major differences in metabolism in whole cells, suggesting that dwarf and control cells are unlikely to differ greatly in flux of metabolites through the citric acid cycle. Thus, this work shows that rotenone is unique (among the several tested mitochondrial inhibitors) in its ability to inhibit WST-1 reduction, and that changes in mitochondrial activity are unlikely to explain the differences between normal and dwarf fibroblasts in resistance to lethal stresses, rotenone, or low glucose condtions.

The results also suggest that differences in glycolytic enzymes, metabolic pathways, and cellular metabolite levels in several conditions are not different between dwarf and control cells. In particular, the data on NAD and its related metabolites show that levels of NADH change as expected in response to low glucose and rotenone, and that dwarf and control levels change equally. Work on NAD metabolites showed a few small, statistically significant differences between dwarf and normal cells in certain culture conditions, but these are inconsistent across conditions and do not suggest a ready explanation for differences in dwarf cell properties. Similarly, there were small changes in levels of ADP and GDP, typically higher in control cells, suggesting that dwarf cells may maintain higher levels of ATP after exposure to various forms of metabolic stress. There was also a small but significant increase in dwarf glucose uptake both with and without serum, which was not accompanied by differences in glucose transporter expression, and thus is likely to involve differential Glut location, i.e. more on the surface of dwarf cells. The increased glucose uptake did not, however, correlate with increases in the expression or activity of glycolytic enzymes, LDH, or GSK-3, suggesting that any differences in glucose metabolism are likely to be small and mainly due to increased glucose concentrations inside the cell. The work did not test the endpoints of glucose metabolism, lactate production and hydrolysis of glucose to water, so it is unknown if the increased glucose uptake correlates with increased glucose metabolism, but the metabolic data suggest dwarf and control cells do not differ in steady state levels in many tested metabolites. Together, the metabolic results from this work suggest few differences in cellular metabolism between dwarf and control cells, with the caveats listed above.

The data also show several properties of the system(s) responsible for reduction of tetrazolium dyes our cultured fibroblasts. These studies show that WST-1 response to glucose withdrawal is fast, measurable at 30 minutes, and non-toxic, and is repeatable using low glucose, rotenone exposure, the glucose analog 2-

DOG, and the glycolysis inhibitor iodoacetate. We also found that glucose metabolism is necessary for WST-1 reduction. This observation does not, however, explain why rotenone can also inhibit WST-1 reduction, because other complex I inhibitors failed to influence WST-1 signals at non-lethal concentrations. The study with other reducible dyes suggests that WST-1 resembles the other extracellular dyes in its response to glucose and rotenone inhibition, and that internal indicator dyes, such as MTT, are unaffected by these inhibitors. Our data agree with previously published work showing that WST-1 is an external dye that is reduced by the plasma membrane redox system (PMRS) (1;2).

Together, this chapter presents data which ruled out several plausible hypotheses about the mechanism of dwarf cellular resistance to metabolic and cytotoxic stresses. The evidence that reduction of WST-1 reflected actions of cell surface electron transport systems turned the focus of our work to studies of the plasma membrane.

Figure 3.1.
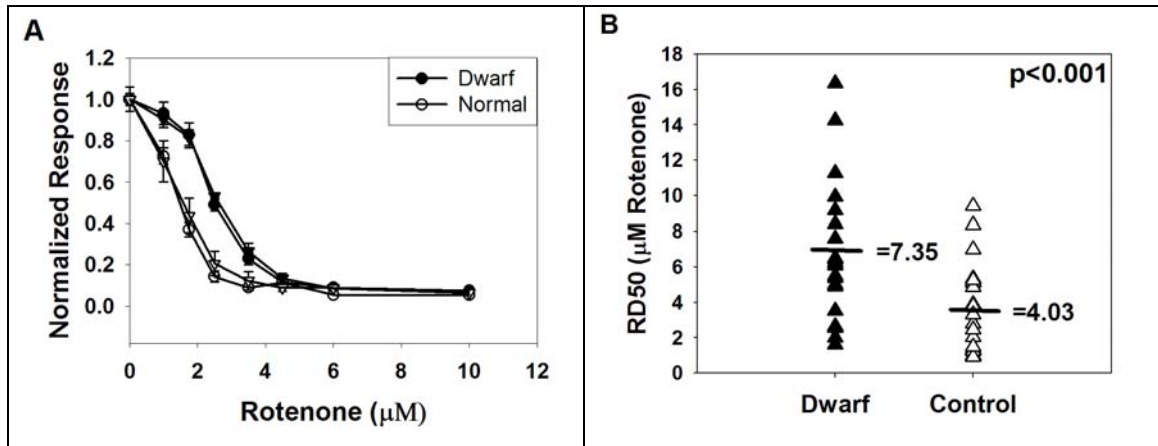
Figure 3.1. Dwarf cells resist the inhibitory effects of rotenone. Figure 3.1 shows the dose response and compiled 50% response doses (RD50) for dwarf (filled symbols) and normal (open symbols) cells exposed to rotenone. Each symbol represents a different cell line taken from an individual mouse, and differences in RD50s were statistically significant by paired t-test.
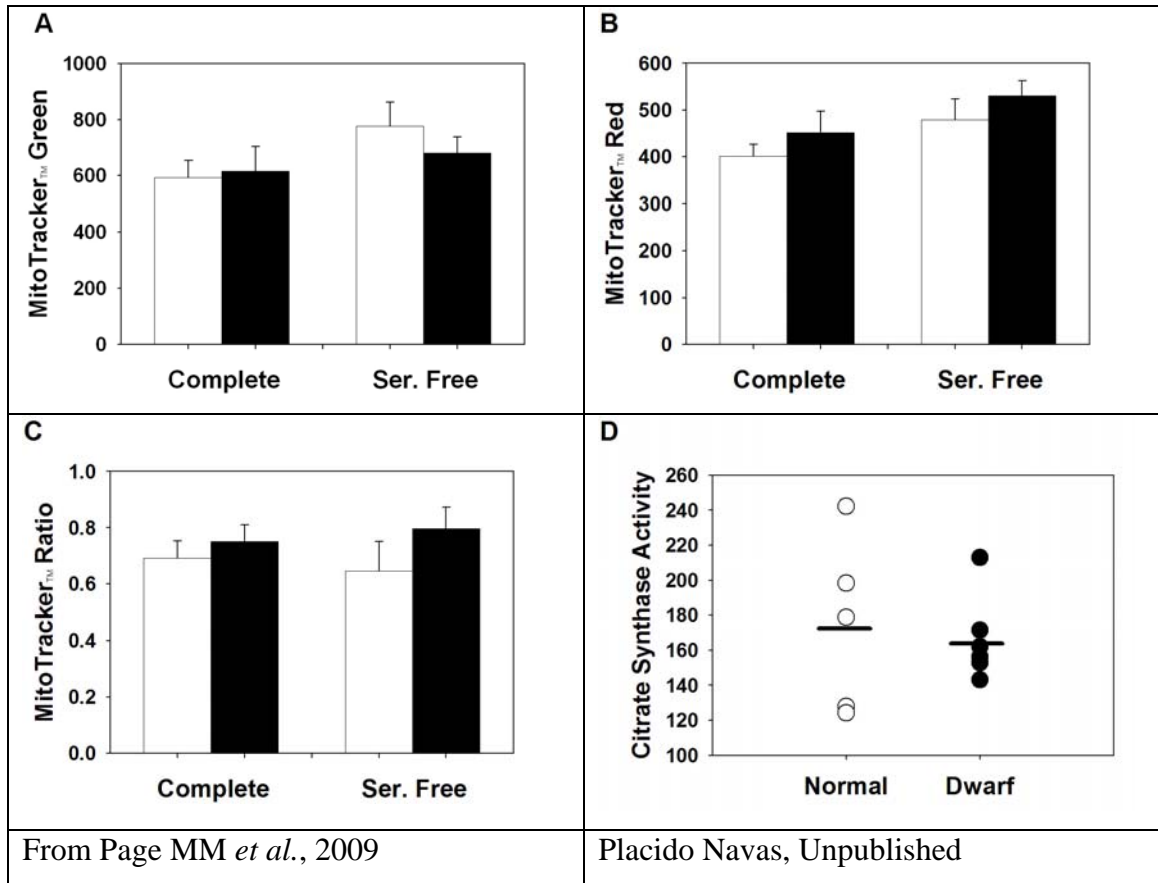
Figure 3.2.
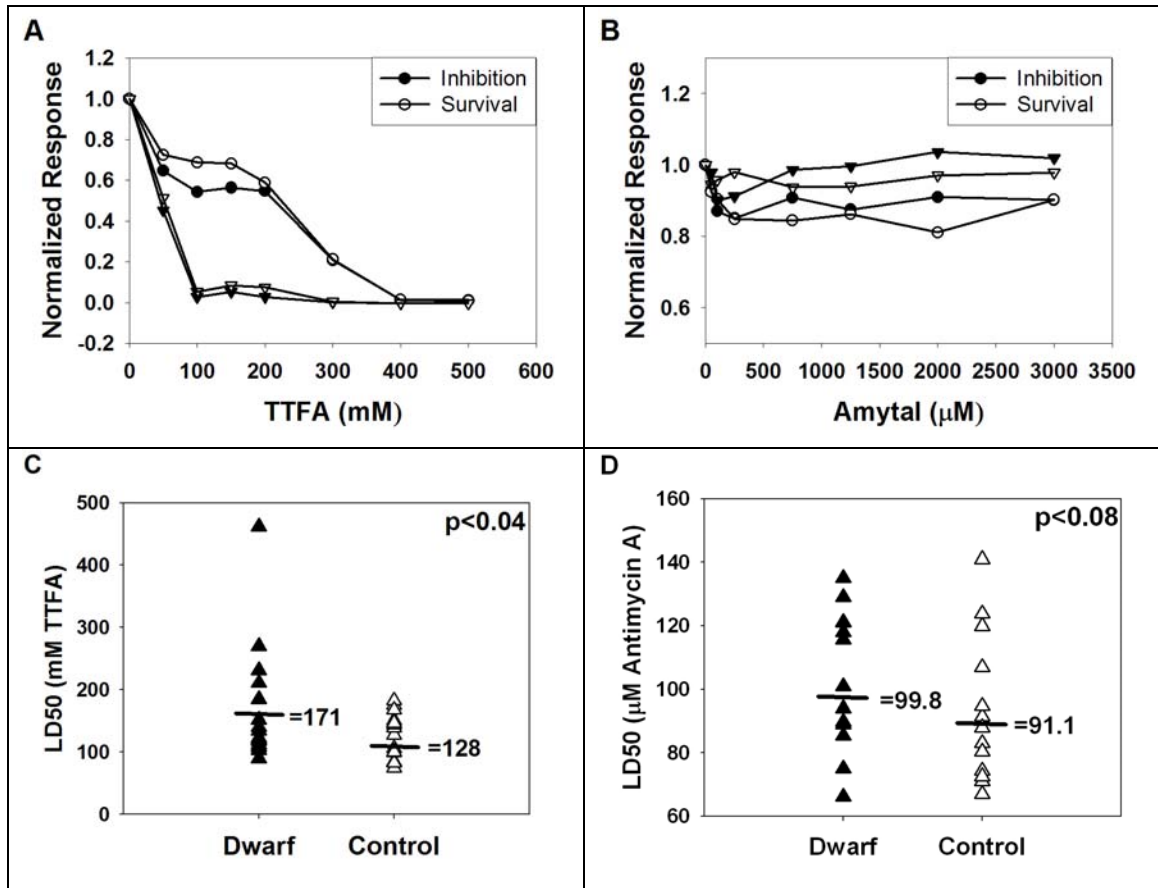
Figure 3.2. Mitochondrial inhibitors do not inhibit WST-1 reduction. Figure 3.2, panels A-B, shows the dose response inhibitory and survival curves for two mitochondrial inhibitors, complex II inhibitor TTFA and complex I inhibitor amytal. Each line represents a different control cell line, with 1 hour inhibitory results in filled symbols and survival in open symbols. Panels C and D show the survival results for TTFA and complex III inhibitor antimycin A, showing that dwarf cells were relatively resistant to the lethal effects of mitochondrial inhibitors, significantly for TTFA and nearly significantly for antimycin A by paired t-test.
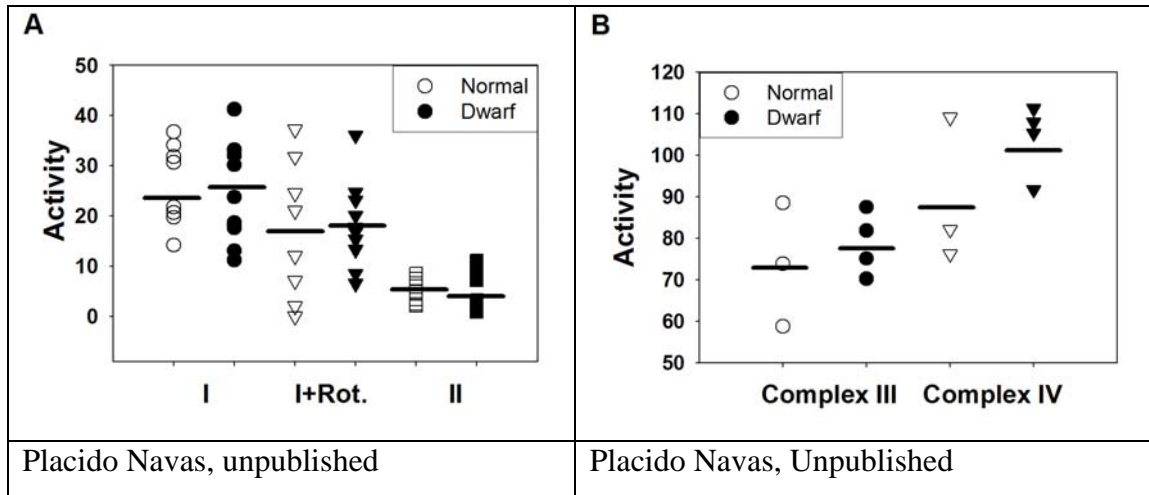
Figure 3.3.

Figure 3.3 panels A, B, C, and D showing MitoTracker Green, MitoTracker Red, MitoTracker Ratio bar graphs for Complete and Ser. Free conditions, and Citrate Synthase Activity scatter plot for Normal and Dwarf.

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Figure 3.3.   Dwarf and control cells have no major differences in mitochondrial number or membrane potential. Figure 3.3 shows the relative quantity and membrane potential of mitochondria in dwarf (filled bars/symbols) and control (open bars/symbols) cells, as measured by MitoTracker fluorescence and citrate synthase activity.   Panels A and D show relative mitochondrial quantities, using MitoTracker green and citrate synthase as markers for total mitochondria.   Panels B and C show the membrane potential, and membrane potential per unit of mitochondrial content respectively. There were no significant differences between genotypes.

Figure 3.4.

Placido Navas, unpublished | Placido Navas, Unpublished

Figure 3.4. Dwarf and control cells have equivalent electron transport. Figure 3.4 shows the relative activities of the complexes of the electron transport chains of cells from dwarf and control mice. Panel A shows the activity of complex I, with and without rotenone (10μM) inhibition, and complex II activity. Rotenone has a significant effect on complex I activity, but there were no genotype differences in the activity of either complex. Panel B shows the activities of complexes III and IV, where there was a trend for increased dwarf activity of complex IV, but no statistical differences.
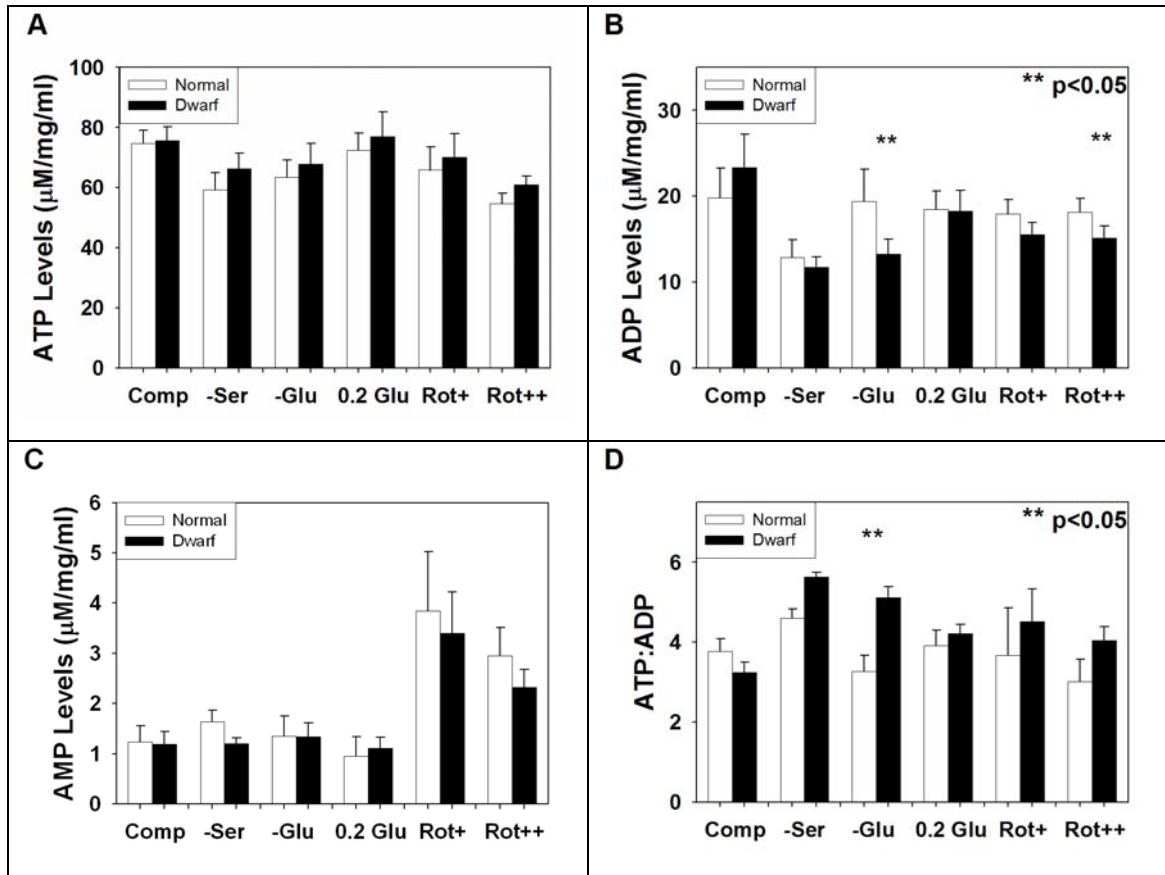
Figure 3.5.
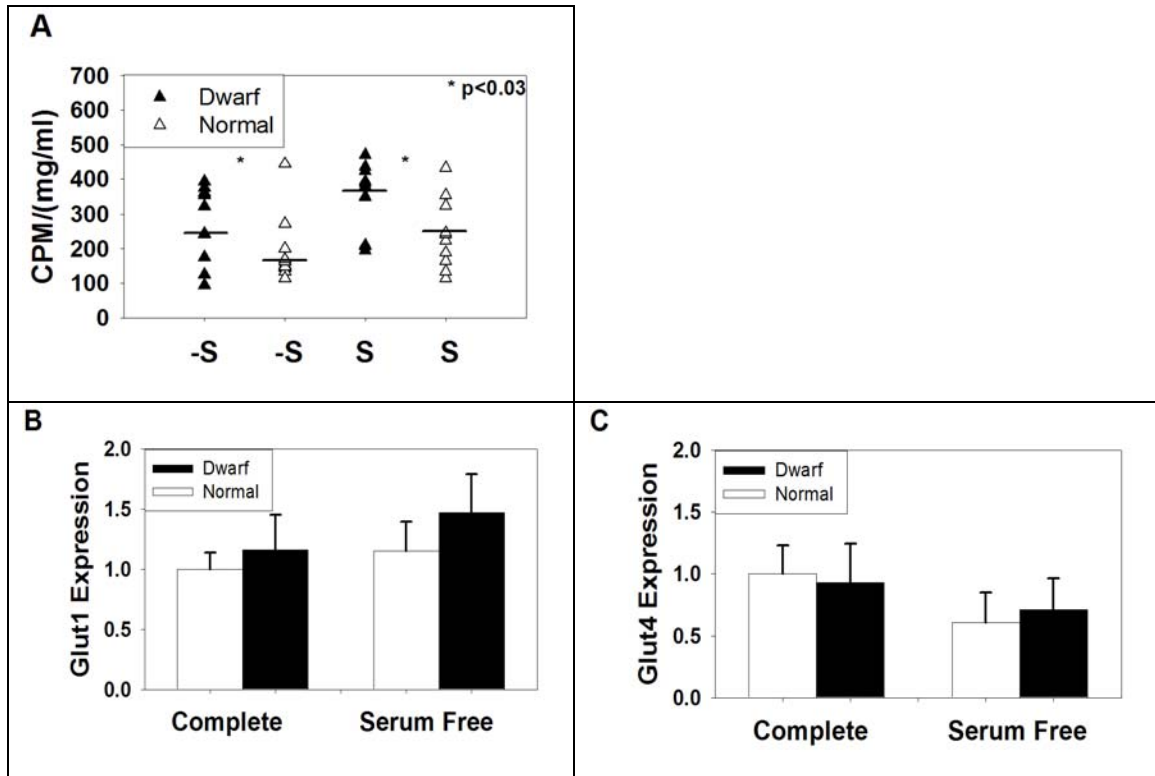
Figure 3.5. Dwarf cells have increased glucose transport with equal Glut expression. Figure 3.5 shows the glucose uptake of dwarf (filled symbols) and control (open symbols), with and without serum (S or -S, respectively), measured by uptake of H^3 2-DOG for 15 minutes. Dwarf cells had a statistically significant increase in glucose uptake as measured by paired t-test. Panels B and C show the expression of glucose transporters Glut1 and Glut4, respectively, for dwarf and control cells with and without serum, as measured by western blot (n=8).

Figure 3.6.
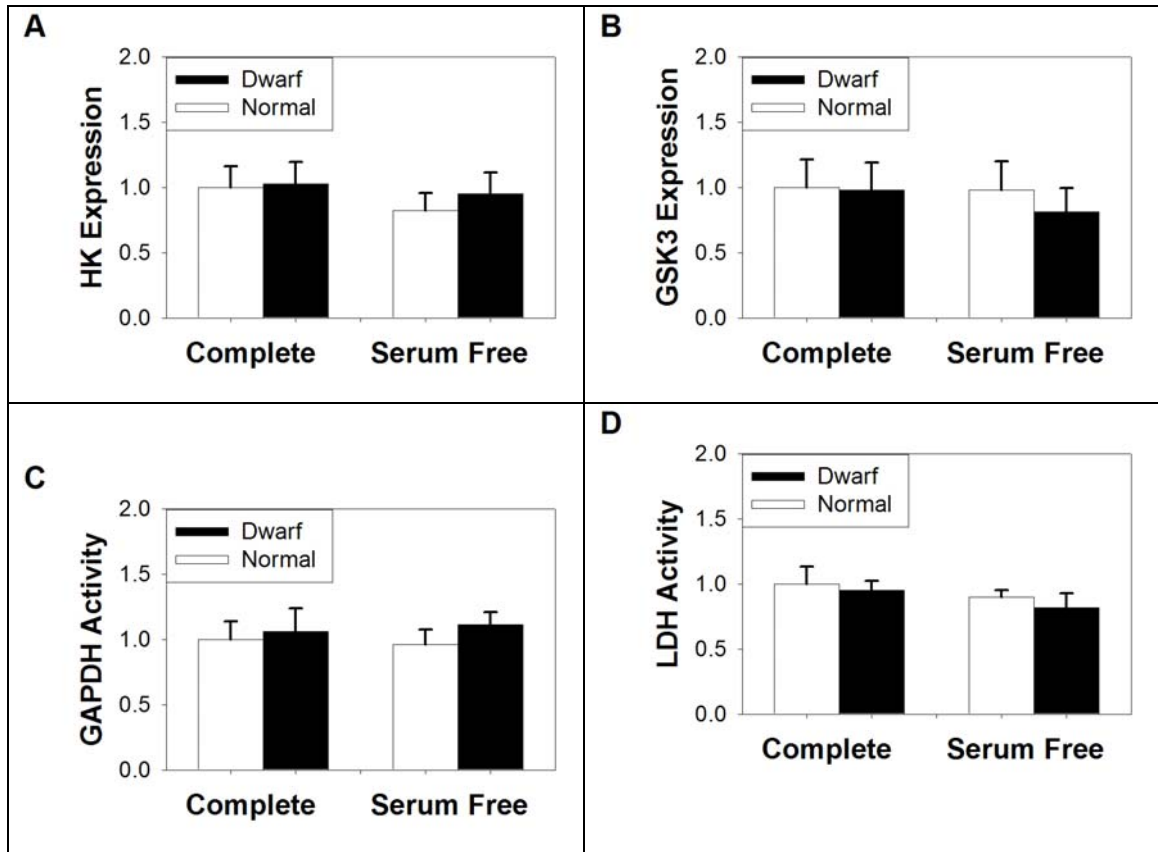
Figure 3.6. Equal expression and activity of metabolic enzymes. Figure 3.6 shows the relative expression (A-B) or activity (C-D) of the metabolically important enzymes, hexokinase I (HK), glycogen synthase kinase-3 (GSK-3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and lactase dehydrogenase (LDH), of dwarf and control cell lysates from control and serum deprived conditions. There were no statistical differences between genotypes or conditions (n=6).

Figure 3.7.

Figure 3.7. Relatively few differences in adenosine phosphates. Figure 3.7 shows the levels of adenosine based phosphates of dwarf and control cells in the following conditions: complete (comp) control condition, serum free (-Ser) medium, glucose free (-glu) medium, low glucose (0.2 glu) medium, low rotenone (Rot+, 10 μM), and high rotenone (Rot++, 50 μM). Panel D shows the ratio between ADP and ATP levels. Metabolite levels were measured by HPLC after acid extraction, and asterisks represent statistically significant differences ($p<0.05$) as measured by paired t-test (n=12).

Figure 3.8.
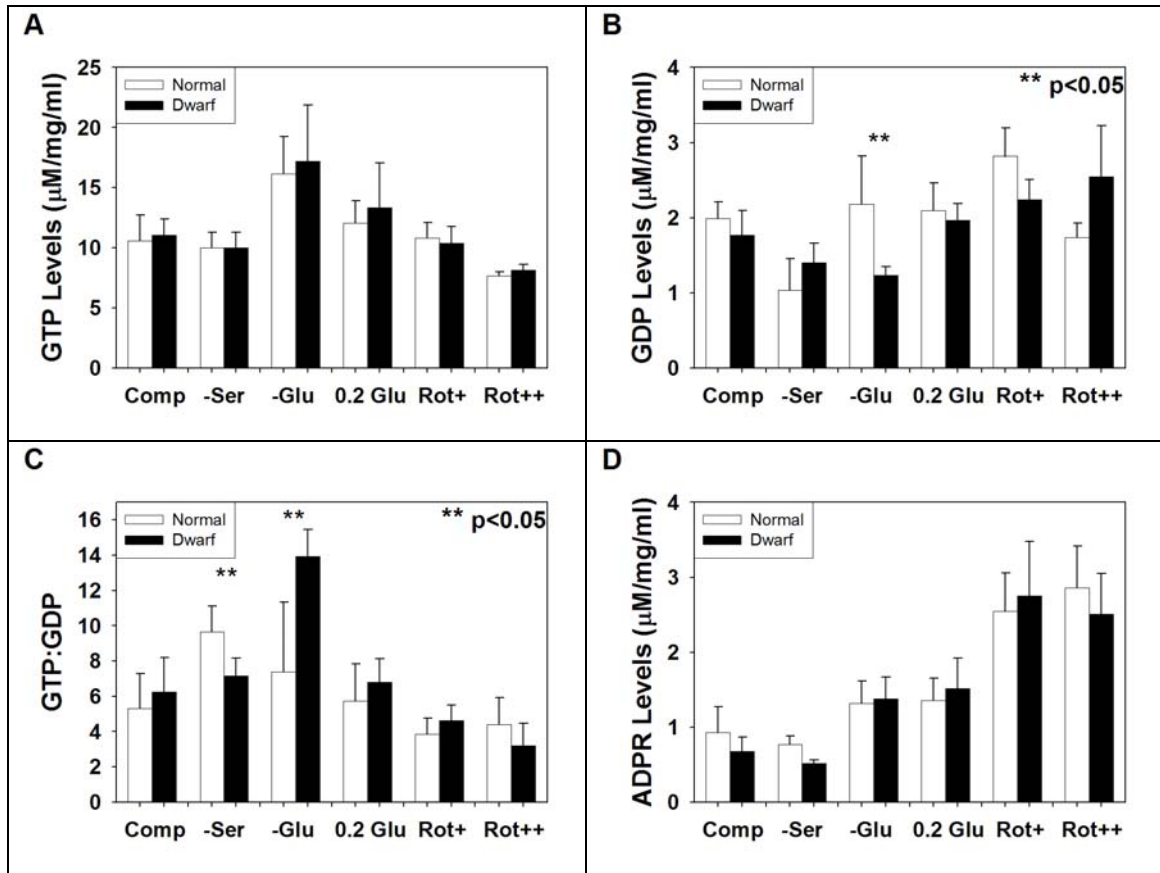

Figure 3.8. Relatively few differences in guanidine phosphates. Figure 3.8 shows the levels of guanine based phosphates and adenosine diphosphate ribose (ADPR) of dwarf and control cells in the following conditions: complete (comp) control condition, serum free (-Ser) medium, glucose free (-glu) medium, low glucose (0.2 glu) medium, low rotenone (Rot+, 10 μM), and high rotenone (Rot++, 50 μM). Panel D shows the ratio between GDP and GTP levels. Metabolite levels were measured by HPLC after acid extraction, and asterisks represent statistically significant differences (p<0.05) as measured by paired t-test (n=12).

Figure 3.9.
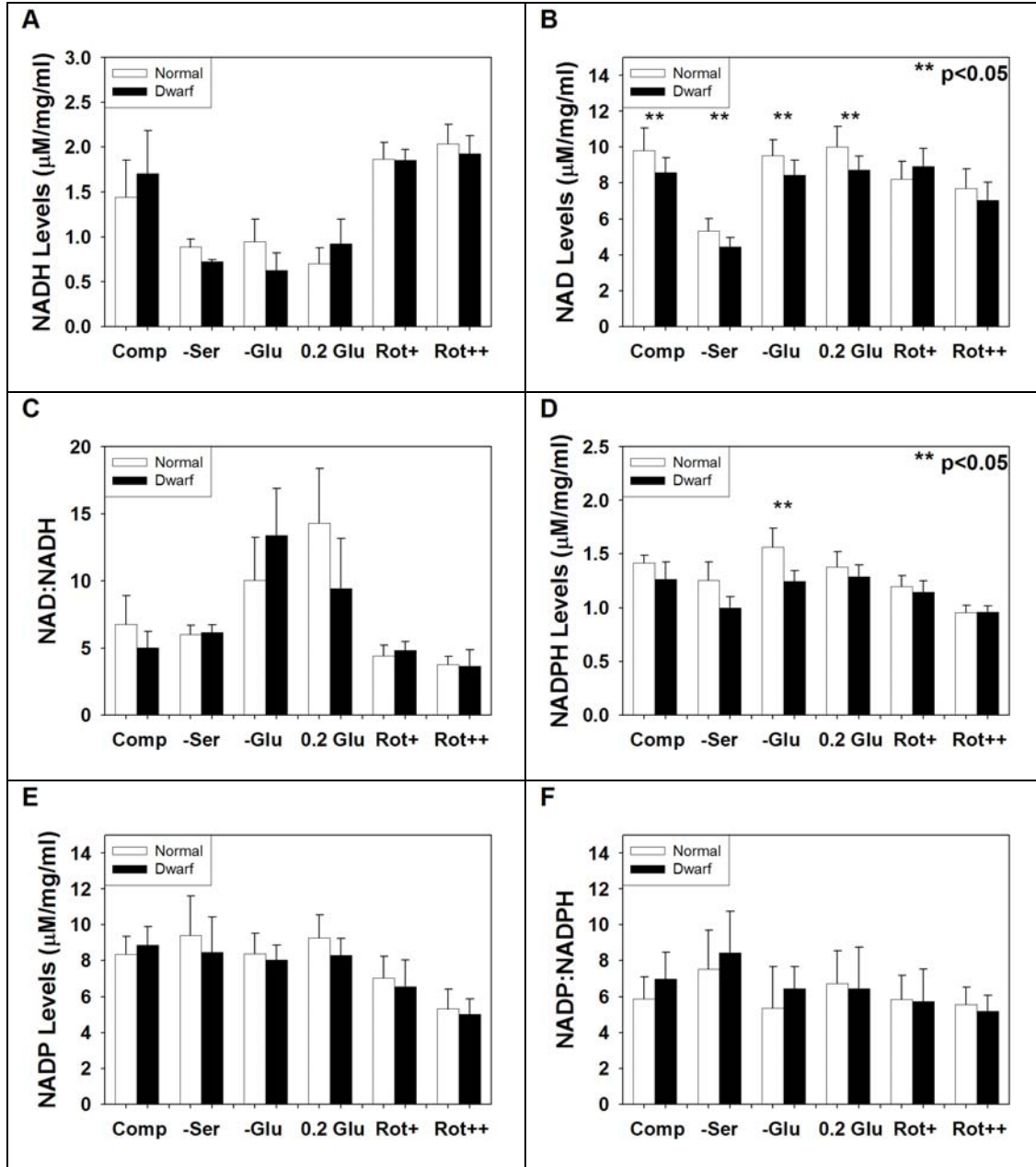
Figure 3.9. No major differences in NAD(P)(H). Figure 3.9 shows the levels of nicotinamide based metabolites of dwarf and control cells in the following conditions: complete (comp) control condition, serum free (-Ser) medium, glucose free (-glu) medium, low glucose (0.2 glu) medium, low rotenone (Rot+, 10 μM), and high rotenone (Rot++, 50 μM). Panel C and G show the ratio between NAD(P) and NAD(P)H levels. Metabolite levels were measured by HPLC after acid (NAD, NADP) or base (NADH, NADPH) extraction, and asterisks represent statistically significant differences ($p<0.05$) as measured by paired t-test (n=12).

Figure 3.10.

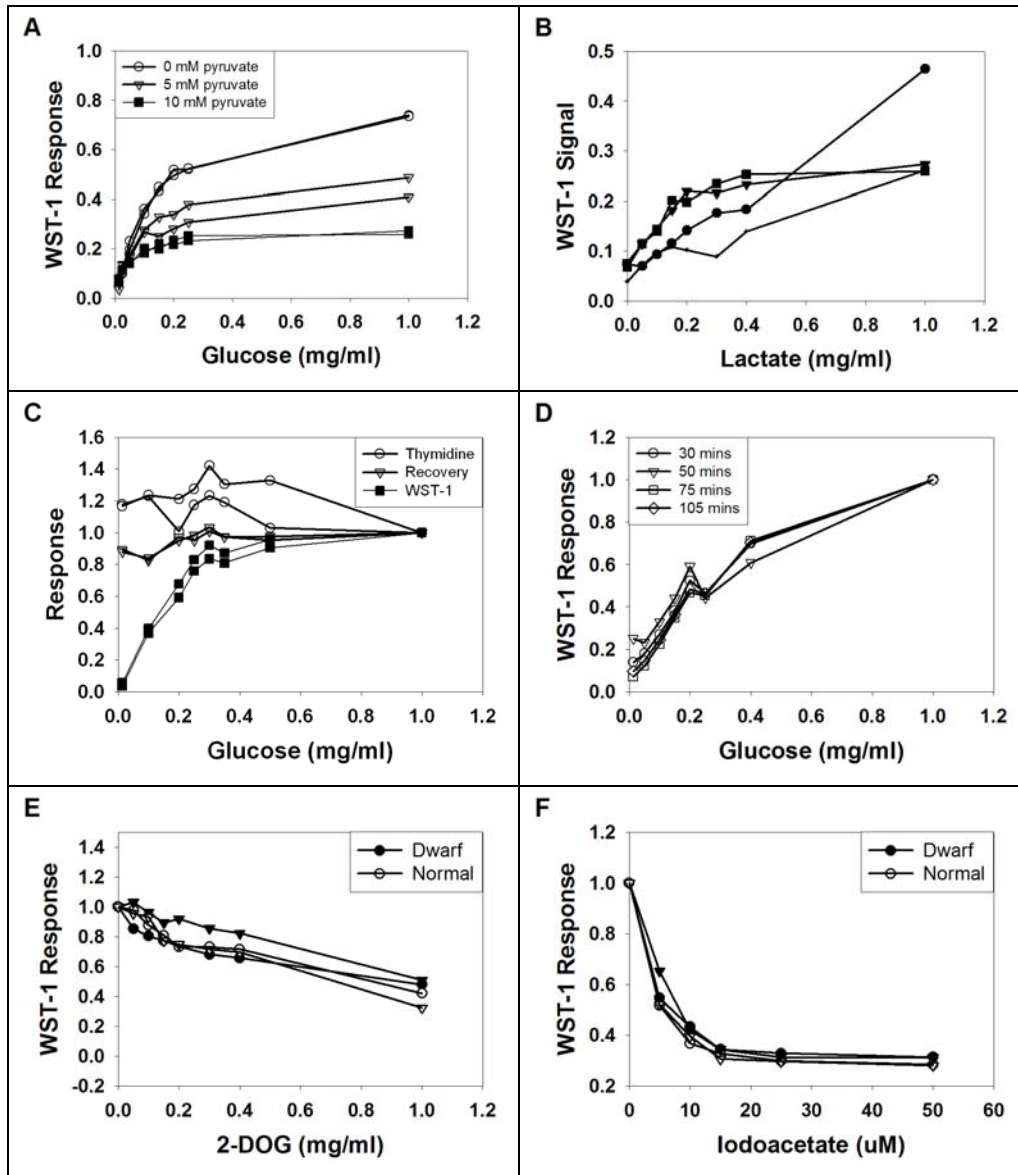**Figure 3.10. WST-1 reduction responds quickly to metabolites, glycolytic inhibitors.** Figure 3.10 shows the WST-1 responses to a variety of metabolites and inhibitors, as well as survival data and time course data for glucose deprivation studies. Panels A and B show the WST-1 response to metabolites pyruvate (with a glucose dose curve) and lactate (no glucose), respectively. Panel C shows the survival of cells in low glucose as measured by thymidine uptake (open circles) and recovered WST-1 signal (gray triangles), along with the inhibitory WST-1 response. Panel D shows the time response of WST-1 inhibition at four time points. Panels E-F are WST-1 dose responses in complete medium of dwarf and control cells inhibited by glucose analog 2-DOG and iodoacetate.

Figure 3.11.
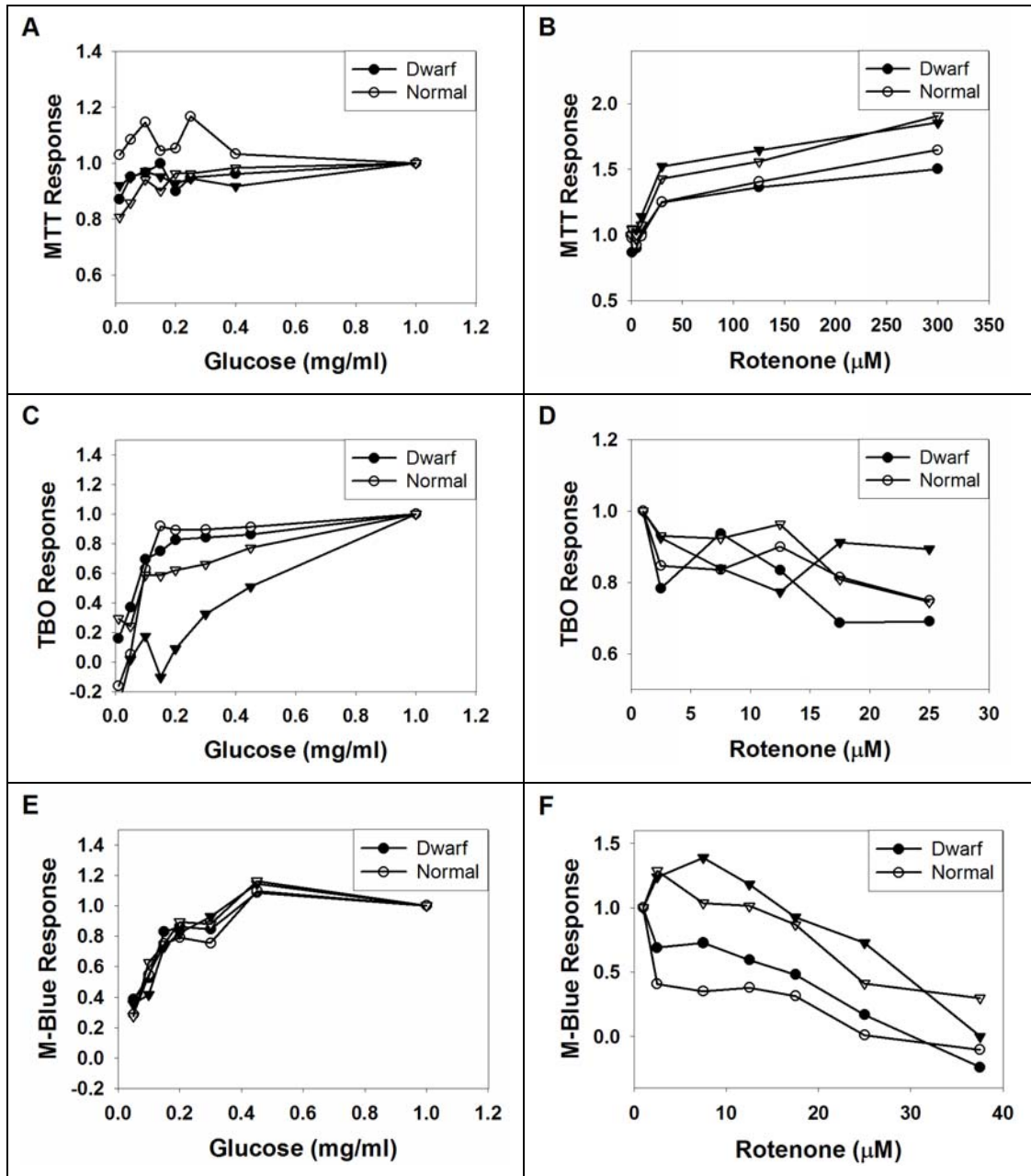
Figure 3.11. **Glucose and rotenone do not affect reduction of the intracellular redox-sensitive indicator dye (MTT).** Figure 3.11 shows the dose response of other reduction-sensitive indicator dyes to glucose deprivation and rotenone exposure in culture. Panels A and B show the response of internally reduced MTT, panels C and D show externally reduced toluidine-blue-O, and panels E and F show externally reduced methylene blue.

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CHAPTER IV

STUDIES OF METABOLIC AND STRESS RESISTANCE IN CELL LINES FROM LONG-LIVED AND SHORT-LIVED RODENTS AND FROM OTHER LONG-LIVED MOUSE MUTANTS

Foreword

The data for this chapter involve stress and metabolic resistance studies, similar to those described in chapter II, that were performed on cells from other animal models. This work serves to verify the relevance of previous work, especially the previous metabolic work, to the aging process. These studies provided additional insight into the implications of the observations on rotenone and low glucose medium reported in chapter II. Most of this chapter is taken from a paper published as Harper JM, Salmon AB, Leiser SF, Galecki AT, Miller RA. Skin-derived fibroblasts from long-lived species are resistant to some, but not all, lethal stresses and to the mitochondrial inhibitor rotenone. Aging Cell. 2007 Feb; 6(1):1-13. Drs. Adam Salmon and James Harper obtained the various cell cultures tested in the paper, and provided culture plates for the metabolic tests, which I performed. Harper, Salmon and Dr. Miller, with statistical help from Dr. Galecki, analyzed the data and produced the figures; I participated in the editing of the final manuscript. This chapter also reports studies of cell lines from other mouse mutants, and in these cases I conducted analyses of cells obtained and provided by Drs. Salmon and Harper, except that the studies of cells from FIRKO mice were entirely my own work.

Introductory findings

In addition to the work on cells from the Snell dwarf mouse, and in collaboration with Andrzej Bartke and Stephen Russell, I performed metabolic and some stress resistance assays on cells isolated from other long-lived mouse stocks. The mice include the Ames dwarf mouse, whose phenotype is nearly identical to the Snell dwarf mouse, the GHRKO mouse, whose similar phenotype was described in chapter I, and the FIRKO mouse, a long-lived model briefly described in chapter I, which has a knockout of the insulin receptor in its fat cells (1). The rationale for using cells from these different types of long-lived mice was to see if the metabolic traits observed in cells from the Snell dwarf mouse were seen in cell lines from other long-lived mutant stocks. Testing the relationship between different mutations and cellular stress resistance will help to interpret other studies of these mutants still under way. Lastly, using the cells from the Ames dwarf mouse allowed us to make sure our results are reproducible in cells from mice maintained by different labs on different backgrounds.

Preliminary results

The results for the Ames dwarf mice, shown in Figure 4.S1 (right side of both panels), suggest that the metabolic phenotypes seen in cells from the Snell dwarf mouse may prove to be present on a similar long-lived model with a different genetic background. While the data set is small and the differences seen do not reach statistical significance, the trend for increased resistance to low glucose and rotenone inhibition in cells from Ames dwarf mice is clearly in the same direction as that of the Snell dwarf mouse. This result was expected, but further solidifies previous work on cells from the Snell dwarf mouse.

Figure 4.S1 also shows results from the GHRKO mouse (left side of both panels). GHRKO mice, like Snell and Ames dwarf mice, have low serum IGF-1 levels. Unlike Snell and Ames dwarf, GHRKO mice have high serum GH levels, but are unable to respond to GH, and they also have normal thyroid hormone

and prolactin levels. Figure 4.S1 shows that they also differ in their metabolic resistance, as it is clear that GHRKO mouse cells are not resistant to the metabolic effects of low glucose, while they appear to resist the effects of rotenone inhibition, although not necessarily to the same extent and not yet reaching statistical significance. This work and data Dr. Salmon has gathered (2) suggest that GHRKO mouse cells are resistant to many of the same stressors as Snell dwarf mouse cells, but are not resistant to cadmium toxicity (2) or the metabolic inhibition caused by culture in low glucose (Figure 4.S1). In chapter II, cadmium resistance was shown to strongly correlate with glucose resistance, which appears to be the case in GHRKO mouse cells, where they resist neither cadmium nor glucose deprivation. This suggests the mechanism for glucose resistance and cadmium resistance may be related, a subject that will be further explored in chapters V and VI.

Figure 4.S2 shows the resistance of cells isolated from FIRKO mice and their controls, tested for both metabolic and cytotoxic stresses. The initial hypothesis in regard to the FIRKO cells was that the knockout of the insulin receptor in fat cells was unlikely to affect the stress resistance of an unrelated fibroblast cell, mainly because levels of growth hormone, insulin and IGF-1 are relatively normal in these mice. The results suggest that fibroblasts from FIRKO cells are relatively resistant to the toxic stresses of cadmium exposure, UV irradiation (nonsignificant trend only), and ER stress from tunicamycin, but do not resist paraquat and peroxide toxicity, or rotenone and low glucose metabolic inhibition. Interestingly, Dr. Amir Sadighi Akha has shown that Snell dwarf fibroblasts have increased susceptibility, not resistance, to tunicamycin and related ER stressor thapsigargin (unpublished), whereas the FIRKO cells resisted tunicamycin toxicity. Currently, not enough is known about the mechanism of these individual stresses to explain why FIRKO mice resist a seemingly random group of stresses, but as more mechanistic information comes forward, it is possible these data will be explained further.

The rest of this chapter involves work with Drs. Harper, Salmon, Galecki, and Miller on skin fibroblasts derived from a wide range of different rodent species, and the little brown bat. The rationale for this work is explained below, and the entire paper is included because while much of the work was performed by my colleagues, the results add significance to the entire project on cellular stress resistance and aging, and include the metabolic stresses introduced in chapter II. The work shows that resistance to effects of rotenone and low glucose are not limited to cells from the Snell dwarf mouse, but are also seen in cells from a variety of long-lived species. Thus, this work helped to encourage the continuation of work on the metabolic differences in cells from Snell dwarf mice in hopes of finding a mechanism that may relate to metabolic resistance, stress resistance, and aging.

Abstract

Fibroblast cell lines were developed from skin biopsies of eight species of wild-trapped rodents, one species of bat, and a group of genetically heterogeneous laboratory mice. Each cell line was 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. Standard linear regression of species-specific life span against each species mean stress resistance showed that longevity was associated with resistance to death induced by cadmium and hydrogen peroxide, as well as with resistance to rotenone inhibition. A multi-level regression method supported these associations, and suggested a similar association for resistance to heat stress. Resistance to cadmium, peroxide, heat, and rotenone was evident after various statistical adjustments for body weight. In contrast, cells from longer lived species did not show significantly greater resistance to ultraviolet light, paraquat, or the DNA alkylating agent MMS. There was a strong correlation between species longevity and resistance to the metabolic effects of low glucose medium among the rodent cell lines, but this test did not distinguish mice and

rats from the much longer-lived little brown bat. These results are consistent with idea that evolution of long-lived species may require development of cellular resistance to several forms of lethal injury, and provide justification for evaluation of similar properties in a much wider range of mammals and bird species.

Introduction

Single gene mutations that extend life span in the nematode worm *C. elegans* often render the worms resistant to multiple forms of lethal injury, including heat, heavy metals, ultraviolet (UV) irradiation, and oxidizing agents including hydrogen peroxide and the free radical generator paraquat (3-5). These observations have suggested that the mutations may prolong life span because they render some or all cells within the worm resistant to various forms of damage from intrinsic or extrinsic agents. A combination of genetic and biochemical analyses has suggested that in these mutants diminished signals from a receptor for insulin-like signals act downstream, via the FOXO family DNA-binding protein DAF-16, to induce a wide range of effector proteins that collectively protect against multiple forms of cellular injury (6;7).

Following these hints, we have previously evaluated the properties of fibroblast cells, derived from biopsies of adult skin, from various stocks of long-lived mutant mice (2;8). The Snell dwarf mouse is homozygous for a loss-of-function mutant of the *Pit1* gene (9), which controls development of the embryonic anterior pituitary. As a consequence Snell dwarf mice have low circulating levels of growth hormone (GH) and its mediator insulin-like growth hormone-1 (IGF-I), thyroid stimulating hormone and its mediators T3 and T4, and prolactin. This set of hormonal changes leads to a reduction in young adult body weight of about 70%, and to an approximately 40% extension of life span (10). We have shown previously that cultured fibroblasts from adult Snell dwarf mice are resistant to death induced by heat, hydrogen peroxide, cadmium, paraquat, and UV, as well as to the DNA alkylating agent methyl methanesulfonate (MMS) (2;8).

Fibroblasts from the Ames dwarf mouse, in which a similar pituitary abnormality leads to dwarfism and extended longevity (11), were also found to be resistant to cadmium, UV, and peroxide (2). In addition, we evaluated cells from growth hormone receptor knock-out (GHR-KO) mice, which resemble the Ames and Snell dwarf mice in their increased life span and in their low levels of circulating IGF-I, but which differ from the Ames and Snell mice in their abnormally high GH levels and relatively normal levels of prolactin and the thyroid hormones (12). We found (2) that fibroblasts from the GHR-KO mice were resistant to UV, peroxide, and paraquat (though not to cadmium), suggesting that development of stress-resistant fibroblasts in these mice may to a great extent represent the effects of diminished circulating IGF-I. In these studies stress tests were conducted on cells grown in vitro for several weeks and through many rounds of cell division, suggesting that resistance to lethal injury represents an epigenetic change, induced in vivo but remaining as a stable cellular property after cell population expansion in serum-containing growth medium. Resistance is absent, or much reduced, in cell lines developed from Snell dwarf mice at ages of 7 days or less (2), showing that the resistance does not depend on possession of the *Pit1*[dw] allele itself, but rather on developmental events that occur in the hormone-deficient mice subsequent to the first week of life.

More recently, we have found (13) that fibroblasts from Snell dwarf mice show a second property of interest: they are relatively resistant, compared to cells from littermate controls, to the metabolic effects induced by the mitochondrial inhibitor rotenone or by culture in medium containing very low glucose levels. The assay involves testing the ability of cells to reduce an extracellular electron acceptor, the tetrazolium dye WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] -1,3-benzene disulfonate). Transferring cultures of control fibroblasts to medium containing either rotenone or drastically lower glucose levels leads to a decline in WST-1 reduction within 15 to 30 minutes. Diminished WST-1 reduction occurs without a corresponding decline in cell viability, and neither rotenone nor low glucose medium leads to a decline in reduction of an intracellular electron

acceptor, the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The effects of low glucose or of rotenone are fully reversible, in that return to control medium after as much as 24 hr promptly leads to full resumption of cell growth and WST-1 reduction. Interestingly, when skin-derived fibroblast from non-mutant, genetically heterogeneous mice were evaluated, those cell lines most resistant to the metabolic effects of low glucose were also significantly more resistant to the lethal effects of cadmium and hydrogen peroxide, suggesting that resistance to both lethal and metabolic agents might be partially modulated by common factors. While it is unlikely that stress resistance of skin cells plays an important causal role in the extended longevity of Snell dwarf mice, these data suggest a model in which hormonal abnormalities in these mice might induce both metabolic alterations and stress resistance in multiple cell types, some of which do contribute to the unusual resistance of these animals to multiple forms of late-life illnesses.

Although single-gene mutations and selective breeding can seldom increase mammalian life span by more than 40% within a species (14), natural selection has produced differences in longevity, among species, of much greater magnitude. Within the mammals, for example, members of some species typically survive only 2 to 3 years under optimal conditions, whereas others typically produce individuals that can attain ages of 50 years old or more (15;16). Even within a single mammalian order, different species can share radically different maximal life spans. The shortest-lived rodents, for example, have maximal life spans of less than 4 years, while other species have recorded life spans of 18 years (porcupine) or 24 years (beaver) in the wild (Steven Austad, personal communication).

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: mouse, rat, red squirrel, white-footed mouse, deer mouse, fox

squirrel, porcupine, and beaver. In our study we also included cell lines from a stock of laboratory-raised ("DC") mice whose genes derive from four commonly used domesticated inbred lines, because of previous observations that wild-derived mice differed from laboratory-adapted mouse stocks in size, hormone levels, maturation rate, and longevity (17). Lastly, we included multiple cell lines from a species of bat (the Little Brown Bat, *Myotis lucifugus*), which is much longer lived than rodents of similar body weight. 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, as well as to the metabolic effects of mitochondrial inhibition.

Methods

Primary and secondary cell cultures and establishment of cryopreserved cell lines. Wild rodents and bats were caught opportunistically by Marc Steinke of Michigan Wildlife Removal and by Phil Myers of the University of Michigan in an area extending approximately 250 miles north and 50 miles south of Ann Arbor, MI. After field euthanization, abdominal skin areas were sterilized with 70% ethanol wipes and biopsies of at least 5 mm by 5 mm in area were obtained and placed in complete media (CM) made of Dulbecco's modified Eagle medium (DMEM, high-glucose variant, Gibco-Invitrogen, Carlsbad, CA) 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) on ice and shipped overnight to our laboratory. For laboratory-raised (DC) mice, animals were humanely euthanized by approved protocols, the abdomen was washed with 70% ethanol, and abdominal skin biopsies of at least 5 mm by 5 mm in area were obtained and placed in CM.

Fibroblast cultures from all biopsies were isolated as we have previously reported (2;8). Cells were harvested by trypsinization for subculturing after the initial

cultures had reached at least 90% confluence. Because some initial cultures grew more rapidly than others, the initial subculturing ("Passage 0") took place between 6 and 9 days of growth after initial seeding. There were no significant differences among species in the mean interval between seeding and the first subculture (not shown). Cell cultures were then fed at day 3 (replacing 2/3rds of the medium), and subcultured at Day 7 at a density of 7.5 x 10^5 in tissue culture flasks of 75 cm^2 surface area to produce Passage 1 cultures. Seven days later, when cells were again confluent, they were harvested and cryopreserved at 10^6 cells/ml in a solution containing CM supplemented with an additional 5% fetal bovine serum and fresh dimethylsulfoxide (DMSO, Hybri-MAX, Sigma) at a final concentration of 10%. The cells were then stored in liquid N_2 for up to 18 months before assessment of stress resistance. Cell aliquots were thawed rapidly using 10 volumes pre-warmed (37º) CM, then centrifuged 5 minutes at 200 g. The cell pellet was resuspended in approximately 6 ml CM and a small volume was used for viability assessment using trypan blue staining. Cells were then cultured at a density of 1 x 10^4 live cells/cm^2 of flask surface area into tissue culture flasks of 75 cm^2 surface area. These "passage 2" cells were fed on Day 3 by replacement of 2/3rds of the medium, and subcultured on Day 7 at a density of 1 x 10^5 cells/cm^2 flask surface area into tissue culture flasks ("passage 3") of 175 cm^2 surface area.

Assessment of fibroblast resistance to lethal stress. For each single set of stress assay experiments, representatives from multiple species (at least 6) were assayed in parallel to minimize the effects of day to day variation. Passage 3 cultures were used to assess resistance to cadmium, paraquat, UV, and MMS, as well as for creating passage 4 cells. Passage 4 cells were then used to assay resistance to H_2O_2 and heat and for the rotenone and glucose resistance assays. Each test procedure began by culturing the cells at a density of 3 x 10^4 cells in 100 µl CM in 96-well microtiter plates in complete medium for 24 hr, followed by a period of 24 hr in medium lacking serum but containing 2% bovine serum albumin (BSA, Sigma) with antibiotics and fungizone at the same concentration

as CM. This pre-culture in serum-free medium was introduced because of our evidence that serum greatly increases stress resistance in mouse cell cultures, obscuring differences in cell stress resistance that can be demonstrated after serum has been removed (8). Tests for resistance to cadmium, peroxide, paraquat, UV, and MMS were performed as previously reported (2;8). For assessment of heat resistance, cells were washed, DMEM prewarmed to 42º was added to each well, and the plates were then sealed with parafilm and placed on a 42º heat block for a range of times. After treatment, cells were washed with 37º 1X PBS, and incubated with 37º DMEM supplemented with 2% BSA, antibiotics and fungizone, and survival was measured 18 hours later using conversion of the extracellular tetrazolium dye WST-1 to its colored formazan product as in (8). All incubations (except for heat) were at 37º in a humidified incubator with 5% CO_2 in air.

Assessment of cell metabolism in low glucose medium. Cells harvested and grown to the fourth passage as above were plated at a density of 3 x 10^5/ml in DMEM with 20% fetal bovine serum with antibiotics and fungizone. After an 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). CM was used as a control. After a one-hour incubation in these media, WST-1 was added, and the extent of its conversion to formazan evaluated 3 hr later as a measure of metabolic activity.

Inhibition of cell metabolism by rotenone. Cells harvested and grown to the fourth passage as above were plated at a density of 3 x 10^5/ml in DMEM with 20% fetal bovine serum with antibiotics and fungizone. After an 18-hour overnight incubation, cells were washed once with 37° C 1X PBS, and incubated in CM with a range of doses of the mitochondrial inhibitor rotenone (Sigma) dissolved in DMSO, or with DMSO only as a control. After a one-hour incubation

in these rotenone doses, WST-1 was added and the extent of its conversion to formazan evaluated 3 hr later.

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 triplicate 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 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.

Estimates of species life span and body weight. Mammalian Species Accounts, published by the American Society of Mammalogists, were used as the source of body weight and estimated maximum life span: *Castor canadensis* (No. 120), *Erethizon dorsatum* (No. 29), *Myotis lucifugus* (No. 142), *Peromyscus leucopus* (No. 247), *Sciurus niger* (No. 479), *Tamiasciurus hudsonicus* (No. 586). In instances where there were no body weight or life span data recorded, or if there was no species account available, these data were provided by Dr. Steven Austad (personal communication). Life span and body weight data for the stock of laboratory-raised DC mice are from a laboratory population maintained by us under standard husbandry conditions (17).

Standard linear regression. Initially, simple linear regression was used to examine the relationship between the reported species maximum life span and the mean LD50 for each of the 8 stressors. A single life span estimate was used for each species.

Multi-level regression. In this analysis we assumed that the species were randomly selected from the entire spectrum of extant species, and consequently treat these data as an example of clustered observations. To evaluate the relationship between LD50 and species life span, we used a mixed effects/hierarchical model, more specifically a linear mixed effects model with a random intercept describing variation between species.   We also evaluated, in parallel, linear and quadratic fixed effects associated with species-specific lifespan, and models allowing heterogeneity of the residual variance, but these models did not improve the fit to the data.

Phylogenetically independent contrasts. Phylogenetically independent contrasts (PIC) were constructed using the PDTREE module in the Phenotypic Diversity Analysis Programs (PDAP) software package available from Dr. Theodore Garland, University of California, Riverside (18-20).   The phylogenetic relationships used to construct the contrasts were compiled using published phylogenies derived from mitochondrial and nuclear gene sequence data (21-30). A copy of the resulting phylogenetic tree is available from the authors upon request.

Regression on body weight residuals. Two sets of models were constructed to determine whether the relationship between life span and stress resistance might simply reflect the well-known relationship between increased life span with increasing body size (31). The first set of models was constructed using simple linear regression to examine the relationship between the stress resistance estimator (i.e., the mean LD50 or ED50 for each stressor) and the residual of life span against species-specific body weight. These residuals were calculated by Dr. João Pedro de Magalhaes using an allometric equation scaling the estimated maximum life span to body weight. The second set of models also used simple linear regression, but in this case the relationship between the residuals of each stress resistance measure (after the adjustment for body weight) and the

residuals of the estimated maximum life span (also after the adjustment for body weight) was determined.

Results

Previous work from our laboratory has shown that primary fibroblast cultures developed from the skin of adult dwarf mice are resistant to at least six forms of lethal injury: heat, cadmium, hydrogen peroxide, UV light, paraquat, and the DNA alkylating agent methyl methanesulfonate (MMS). In addition, cells from dwarf mice show a metabolic abnormality: they are relatively refractory, compared to cells from littermate controls, in response to culture conditions that inhibit the reduction of extracellular electron acceptors, such as growth in medium that is low in glucose or which contains non-lethal levels of the mitochondrial inhibitor rotenone. Because mammals, including the rodents that are the particular focus of this study, have evolved life spans that vary over at least an order of magnitude, we wished to determine if fibroblasts from a range of different species show a similar pattern of resistance to lethal stresses and metabolic inhibitors. To investigate this question, we assayed cell lines developed from a convenience sample of mammalian species shown in Table 4.1.

Several features of the experimental design and analysis deserve explicit justification. Two kinds of mice (*Mus musculus*) were used in this study, and treated separately for analysis. Laboratory mice were the product of a four-way cross among commonly used inbred strains; these animals were bred in the laboratory and housed in vivarium conditions until euthanized for study. A second set of samples was derived from wild-trapped mice. Because long-term domestication and inbreeding lead to dramatic changes in behavior, developmental rate, body size, and longevity (17;32;33), we thought it of interest to include samples from both kinds of mice in our study for comparison. All other samples were from wild-trapped animals. The age of these animals is not known, although by size each donor was judged to be young adult. We did not

consider the sex of the donor at any point in the analysis; all of the DC laboratory mice were males, but the sex of the wild-captured animals was not consistently recorded. All of the wild-trapped animals were trapped within 250 miles of Ann Arbor, MI, thus providing some uniformity of adaptation to specific climate conditions. Lastly, we included in our study set one species of bat in addition to the 8 species of rodents, because of previous work (31;34) suggesting that bats were particularly long lived compared to non-flying eutherians of similar body size.

The design was thus compatible with several analytical strategies addressing a set of related and overlapping issues, including comparisons among all species; among rodents only; among wild-trapped rodents only; or between pairs of species of particular interest. We also considered, and will discuss below, analytical methods that adjust in various ways for phylogenetic relationships or for inter-species differences in body weight.

Figure 4.1 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 4.1 (i.e. treating laboratory mice and wild-trapped mice 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. The results of this analysis suggest that for this group of samples maximum life span is positively correlated with fibroblast cell line resistance to cadmium and hydrogen peroxide. 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.

Skin-derived fibroblasts from Snell dwarf mice are also resistant to the metabolic effects of the mitochondrial inhibitor rotenone and of low-glucose culture media (13). Although neither low glucose medium nor rotenone leads to cell death in the conditions used, both lead rapidly (within 15 minutes) to a reversible inhibition of the ability of mouse fibroblasts to reduce extracellular electron acceptors such as the tetrazolium dye WST-1. Interestingly, a test of cell lines from non-mutant genetically heterogeneous laboratory mice showed that those individual mice whose cells were most resistant to the lethal effects of cadmium and hydrogen peroxide were also most resistant to the metabolic inhibition caused by low glucose conditions, suggesting that common cellular factors might contribute to resistance to both lethal and non-lethal agents. Because cells from long-lived species were relatively resistant to cadmium and hydrogen peroxide, we tested these cells for resistance to low glucose medium and rotenone as well. Results are shown in Figure 4.2, and show that cells from long-lived species are relatively resistant to the inhibitory effects of rotenone ($R^2 = 0.58$, $p < 0.02$). The data from the low glucose tests were ambiguous: when all species were included, $R^2 = 0.32$, with $p = 0.09$; when only rodents were evaluated, $R^2 = 0.7$, $p < 0.005$.

Secondary analyses. We also conducted a series of secondary analyses. Some were conducted to evaluate specific contrasts of interest, and some of these were motivated by a desire to test our conclusions for robustness to the arbitrary design decisions, such as the inclusion of a non-rodent species and the decision to treat laboratory mice and wild-trapped mice separately. Other analyses were undertaken to evaluate alternate regression approaches that are favored by some, but not all, experts in inter-species contrasts.

Table 4.2 presents the results of three different regression analyses of a data set containing all of the tested species (including laboratory and wild mice as separate entries, and including the bat species). The first column shows standard linear regression results, regressing the mean LD50 (or ED50 for non-

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lethal stresses glucose and rotenone) value for each species against the estimate of maximum life span for each species. These calculations were presented above in the context of Figure 4.1.

The second column shows a different approach, in which each individual donor was included in a two-level regression, similar to the "nested regression" models sometimes used in other contexts. The calculation separated overall variation into "between-species" and "within-species" components, in a linear mixed effect model, and made the assumption that the sample of species were randomly selected from a larger population of species from which data might have been obtained. The tabulated p-value represents the significance of the "across-species" term in the regression model. 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 $p = 0.02$.

There is controversy (discussed below) about the appropriateness of including information about phylogenetic relatedness for evaluation of inter-species effects in small data sets of this kind. Nevertheless, we used a popular method (35;36) for evaluating phylogenetically independent contrasts within this data set, and the results are shown in the last column of Table 4.2. The association of cadmium and peroxide resistance with species longevity remains significant after adjustment in this way; the rotenone association is no longer significant ($p = 0.08$).

The analyses shown in Figures 4.1 and 4.2 and in Table 4.2 make use of information from all of the cell lines we tested, including cells from laboratory mice and from a bat species. We have also performed regression analyses using a subset of the data limited to wild-trapped rodents only (with both bats and laboratory mice excluded). Table 4.3 shows regressions for this truncated data

set. 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 4.2 are no longer statistically significant, and testing the robustness of our conclusions clearly will require additional information from a wider range of species.

Some authorities (37) have suggested that regressions of longevity on cellular parameters often reflect the well-documented trend towards longer life spans among larger mammals (34), and have recommended that regression analyses be adjusted for species differences in body weight. Table 4.4 shows the outcome of two such adjusted regression studies. In each case the dependent variable is not life span per se, but the residual of life span against species specific body weight. These residuals were calculated by Dr. João Pedro de Magalhaes, using an extensive data base of life span and body weight values for 1334 mammalian species (38). The first data column shows regressions in which LD50 (or ED50, for glucose and rotenone) are regressed against this weight-adjusted life span residual. There are significant associations for cadmium, peroxide, heat, and rotenone in this set of models. The second column shows a similar approach, in which the residual of life span (regressed against body weight) is compared to the residual of the LD50 value, itself regressed against body weight for those species for which we have stress resistance data. Here, too, significant associations are seen for cadmium, peroxide, heat, and rotenone.

Lastly, we used subsets of our data to consider a small number of specific pairwise comparisons of particular biogerontological interest. The first three columns of Table 4.5 evaluate whether laboratory mice differ from wild-trapped mice in the resistance of their cells to the agents under consideration. Cells from wild-trapped mice are significantly more resistant to MMS, heat, and UV light, and significantly more resistant to the effects of low glucose medium. There is no evidence of preferential resistance to peroxide or paraquat. Cells from wild-

trapped mice were, unexpectedly, dramatically and significantly more sensitive to cadmium toxicity than were cells from laboratory mice. Cells from wild-trapped mice were 3.4-fold more resistant to rotenone-mediated inhibition, but there was a great deal of scatter among individual animals, and the difference was not statistically significant ($p < 0.07$).

There is also considerable interest in the question of why flying mammals, such as bats, are typically so much longer lived than non-flying animals of similar size (31;34). We therefore include in Table 4.5 a comparison of cells from the little brown bat to cells from (wild-trapped) mice and rats for each of the agents studied. Bat cells are significantly more resistant than mouse or rat cells to cadmium, hydrogen peroxide, and heat, among the lethal stresses, and also significantly more resistant to the non-lethal mitochondrial inhibitor rotenone. The difference in resistance to MMS reaches statistical significance for rat cells ($p = 0.03$), but not for mouse cells ($p = 0.06$). Unexpectedly, cells from this bat species are significantly more sensitive to paraquat than cells from wild-trapped mice. There are no differences between bats and either of these two rodents in resistance to UV or to low glucose medium under our conditions.

Discussion

Primary dermal fibroblast cell lines isolated from 8 species of rodent (house mice, deer mice, white-footed mice, Norway rats, red squirrels, fox squirrels, beavers and porcupines), and a single species of bat (little brown bat) were used to examine the relationship between life span and resistance to many kinds of cell injury. Previously, we have shown that cell lines from long-lived Ames dwarf (*df/df*), Snell dwarf (*dw/dw*), and growth hormone receptor knockout (GHR-KO) mice each exhibit increased resistance to multiple cytotoxic agents *in vitro* relative to cell lines derived from non-mutant controls (8;39). These findings led us to hypothesize that differences in stress resistance of adult dermal fibroblast cells might serve as an indicator to cellular properties involved in disease

resistance and longevity in intact organisms, even though it is unlikely that the dermal fibroblasts themselves play an important role in life span determination.

Expert opinion is divided on the question of how best to evaluate hypotheses about distribution of biochemical and cellular phenotypes across a range of species that differ in typical or maximal longevity. Some have argued (37) that such analyses are invalid unless the traits are first adjusted for body weight, to avoid reporting relationships that merely represent correlations among traits known to depend heavily on body size and its associated thermogenic metabolic demands. This notion, while important to consider, would eliminate from consideration nearly all cellular properties that lead to delayed aging in mammals, because large species of mammals are, in general, longer-lived than small species. Some experts maintain that cross-species comparisons require adjustment for the degree of phylogenetic independence among the species evaluated, while other authorities include such adjustments only when there is a demonstrable and statistically significant effect of phylogenetic relatedness in the data set itself (40-42). Assessments of the strength of this "phylogenetic signal" are, however, difficult to make with data sets containing fewer than about 20 species. Nor is there a consensus about how to analyze data sets in which estimates from each species vary in their precision, in our case because some species are represented by multiple individuals, but sometimes by only one specimen. Because of these uncertainties, we have presented several different analyses of the same data set so that proponents of each approach can evaluate the strength and weaknesses of the evidence available to us. Fortunately, most of the conclusions we reach are robust to the analytical approach; we point out exceptions where these seem of interest.

Considering the entire set of species, long lifespan is associated with relative resistance to cadmium, H_2O_2, and the non-lethal inhibitor rotenone. There are also suggestions of a similar relationship for heat (p = .053) and MMS (p = 0.08). Although to be conservative we used two-tailed p-values as our significance

criteria, we note that in each case the direction of the regression is consistent with our original working hypothesis, i.e. that cells from longer-lived species would be more resistant to the lethal stresses and non-lethal metabolic inhibitors, as are fibroblasts from long-lived mutant Snell dwarf mice. When multi-level regression is used to take account of differences among the species in number of individuals tested (and differences in variance within a species), the associations for cadmium, H_2O_2, and rotenone remain significant, and in addition heat stress has a significant association at $p = 0.02$. When the data are evaluated using the method of phylogenetically independent contrasts, the cadmium and H_2O_2 effects remain significant, despite the loss of statistical power in this approach; the rotenone association remains suggestive at $p = 0.08$. Thus despite the small number of species and individuals examined, we see an association for cadmium and H_2O_2 that is robust across analytical methods, and have varying degrees of support for parallel hypotheses for rotenone, heat, and perhaps MMS.

In addition to these regressions of cellular traits against life span, we also performed a regression based on the residual of life span adjusted for body weight, and a regression in which both the cellular trait and life span were body-weight adjusted (see Table 4.4). Both approaches revealed significant associations for cadmium, H_2O_2, heat, and rotenone. It thus seems unlikely, in this data set, that the differences among species in resistance to these four conditions reflect variation with body size alone.

The main dataset includes data from one non-rodent species (the little brown bat), and also from a group of laboratory-adapted mice. The decision to include both laboratory-adapted mice as well as those derived from recently-trapped wild progenitors was motivated by the finding that wild-derived mice live longer, are smaller, mature more slowly, and have lower levels of several hormones compared to typical laboratory mice (17). To see if our principal findings depended on the inclusion of bats and laboratory-adapted mice, we evaluated a reduced data set consisting of wild-trapped rodents only. In this truncated data

set we see a strong correlation for resistance to low glucose media, with $R^2 =$ 0.81 (p < 0.002) using standard regression, and similar, significant associations with the other two analytical methods. Indeed, among the wild-trapped rodents, the association between longevity and glucose effect is stronger than the associations with any of the tested lethal agents. The relationship of cadmium to life span was also supported in this subset of the data, and the multi-level regression indicated associations for heat and rotenone as well.

It is important to note that although WST-1 is used in tests of both lethal stresses (cadmium, heat, H_2O_2) and in the evaluation of responses to low glucose, the decline in WST-1 reduction under low glucose conditions is not accompanied by cell death. The lower level of WST-1 reduction seen in low glucose conditions seems likely to reflect alterations in metabolic pathways needed to produce the reducing equivalents for WST-1 cleavage at the plasma membrane (43;44). Fibroblasts remain fully viable even 48 hr after initiation of glucose deprivation, with full recovery of WST-1 metabolic activity 1 hr after return to regular growth medium with a glucose concentration of 4 mg/ml. In addition, we found that glucose withdrawal for a period of 1 hour or 24 hours had no effect on the levels of DNA synthesis in mouse fibroblasts as measured by radiolabelled thymidine incorporation (not shown). Similarly, the effects of rotenone on WST-1 signal inhibition are fully reversible at the doses used. For example, when cells are exposed to 5 µM rotenone for 4 hr and then allowed to recover in rotenone-free medium overnight, cell viability as measured by WST-1 reduction returns to a level greater than 80% of that seen in untreated cells.

In a study of fibroblasts from individual, genetically heterogeneous laboratory mice (13), we found a significant correlations between resistance to low glucose and resistance to death induced by H_2O_2 (R= - 0.58, p < 0.001) and between glucose and cadmium resistance (R = -0.53, p = 0.006), but not between glucose and the other lethal agents tested. Resistance to lethal injury induced by cadmium or H_2O_2, as well as resistance to the inhibitory effects of low glucose

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and of rotenone are also seen in cell lines from Snell dwarf mice (13). We thus speculate that the biochemical alterations that mitigate the effects of rotenone or glucose withdrawal on cellular metabolism overlap closely with those that impart resistance to cadmium and H_2O_2, whether these differences are caused by the hormonal milieu of dwarf mice, or instead are the result of co-evolution during the course of divergence among mammals of aging rate and longevity.

Overall, our data are consistent with other studies demonstrating an association between mammalian life span and cellular physiology *in vitro*. The closest correspondence is to the study of Kapahi et al. (45), which demonstrated that resistance of fibroblasts to oxidative stressors was positively correlated with mammalian longevity across several taxonomic orders. Kapahi evaluated cells from hamster, rat, marmoset, rabbit, sheep, pig, cow and human, and showed significant associations with maximal life span for H_2O_2 and paraquat in addition to sodium arsenite, *tert*-butyl hydroperoxide, and sodium hydroxide. Hydrogen peroxide and cadmium are both known to induce cytotoxicity via reactive oxygen species (46). Likewise, several other reports, usually involving fewer species, have demonstrated associations involving oxidative stress, antioxidant capacity and maximum life span (Tolmasoff et al., 1980; Sohal et al., 1990; Ogburn et al., 1998; Ogburn et al., 2001). Thus in our study 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. Unexpectedly, however, 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. This suggests that there is a division in the pathways involved in mediating the resistance to particular types of oxidative stress, i.e., paraquat-induced production of superoxide at intracellular sites (47;48) as contrasted to damaged induced rapidly by contact with extracellular H_2O_2, and that production of long-lived species of rodents may not require alteration of the defenses tested by paraquat in culture.

We find no evidence for an association between longevity and resistance to UV light in any of the data sets or with any of the analytical methods used. Other groups have suggested that the repair of UV-induced DNA lesions is correlated with longevity across mammals (49-51), and also within a single order, the primates (52). Within the rodents, however, the data are sparse and ambiguous (53), and some studies have found no evidence for a relationship between life span and UV resistance among species of mammals (54) or non-mammalian vertebrates (55). Furthermore, rodent cells are unusual in that the repair of UV-induced DNA damage does not correlate with cell survival (reviewed in (56). Thus despite our negative evidence in the UV data set, we think it would be informative to explore differences among these and other mammalian species in the capacity of fibroblasts to repair UV or MMS-induced DNA lesions.

Our study design has some features that weaken the generality of our conclusions. For the wild-trapped animals, we do not know the exact donor age, although body size and conformation suggested that the animals were young adults. We did not control the time of year at which samples were taken, except that all individuals were captured between May and October. We did not control for donor sex or reproductive status, although we note that our studies of laboratory mice have given no indication of gender effects on fibroblast stress resistance (Salmon, unpublished). We did, however, control two variables known to influence properties of cultured fibroblasts: (a) each biopsy was taken from the same place (lower abdomen), and (b) cells were tested after the same number of population doublings, and after one cycle of cryopreservation.

Species-specific phenotypes reflect the evolutionary selection pressures unique to each species, as well as the constraints imposed by a shared evolutionary history with other members of a phylogenetic lineage (31;36;57). We sought to diminish the confounding effect of evolutionary history by focusing most of our attention on a single order of mammals, the rodents, unlike other studies in which

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familiar pet or domestic species, from multiple mammalian orders (or sometimes even distinct classes of vertebrates) were evaluated. We also included data on the Little Brown Bat, a member of the order *Chiroptera*. Bats are comparable in size to house mice, but are exceptionally long-lived for their size, even after accounting for effects of hibernation (58). Our working hypothesis was that cells from bats would be more resistant to each agent than cells from mice or rats, and we see in Table 4.5 that this idea was confirmed by data on cadmium, H_2O_2, MMS (significant for rats, $p = 0.06$ for the contrast with mice), heat, and rotenone. Unexpectedly, bat cells seem to be more sensitive to paraquat than cells from mice ($p = 0.03$) and, perhaps, rats ($p = 0.08$), another indication that sensitivity to paraquat and to H_2O_2 may be governed by different cellular defense mechanisms. We have only evaluated cells from one bat species, and the hypothesis that in general bat cells are more resistant to these agents than cells of rodents will require further work. Similarly, it would be of interest to determine if species specific longevity was correlated with cellular resistance to these stresses among the different *Chiropteran* species.

The contrast between laboratory and wild-derived mice, also shown in Table 4.5, gives some indication about the speed with which differences in fibroblast stress resistance can be produced by evolutionary pressures. The common laboratory stocks from which the DC mice are derived emerged from wild populations only in the last 100 to 200 years (59), and during that time have adapted to selective pressures of laboratory vivaria by changes in body size, litter size, hormone levels, activity patterns, maturation rates, and multiple behavioral traits. We can be confident that many of these traits are determined by genetic differences between laboratory and wild mice, because the traits remain distinct in the second generation offspring of wild-captured mice, whose lives, like those of their parents, have been spent entirely in the laboratory environment (17;32;33). Fibroblasts from wild mice are significantly more resistant than those of laboratory (DC) mice in responses to MMS, heat, UV, and low glucose media, resembling cells from long-lived mutant mice in each of these respects, and show

a similar trend (p = 0.07) towards rotenone resistance as well. These data suggest that resistance to some of these agents can evolve within a few hundreds of years, and may not require the millions of years that typically separate distinct species. Curiously, wild mouse cells are not more resistant than laboratory mouse cells to cadmium or to H_2O_2, the two agents for which the evidence for inter-species correlation is strongest. In fact, cadmium resistance is dramatically and significantly higher in cells from laboratory mice than in cells from wild-trapped mice; we speculate that this may reflect selection, under laboratory conditions, for resistance to heavy metals that may be a more significant reproductive hazard in the laboratory than in most natural settings. The differences in the spectrum of stress agents that discriminate long- from short-lived species, and those which discriminate the short-lived laboratory stock from wild mice may reflect the time scale over which evolutionary pressures act to produce speciation, elements of the mouse genome that are more or less susceptible to rapid selection, and the peculiarities of the laboratory environment.

Our findings are also limited in scope in two critical dimensions: we have examined only a single cell source (the skin of adults), and most of our data come from a single order of mammals. Meeting the first concern will require development of new, carefully validated methods to study stress resistance of other cell types, such as vascular endothelial cells, lymphocytes, neurons, muscle cells, etc. Developing methods to culture other differentiated cell types from multiple species would require a good deal of effort. Long-term, transformed cells are often convenient for serial studies, but studies of primary, early-passage cells (as in our own study) helps to avoid the artifacts of in vitro selection for cell resistance to stress. It now seems clear that the oxygen concentrations (20%) used for typical in vitro cell cultures can induce mutations and growth abnormalities in mouse cells that do not occur when the cells are grown at the lower oxygen concentrations (e.g. 3%) characteristic of most post-capillary beds in intact animals (60-62). Attempts to study resistance of specific

cell types in intact animals also pose serious technical obstacles, beyond the scope of this discussion.

The second weakness – the lack of species diversity in our collection – may be somewhat easier to fix. 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, we think, be of even greater interest to see if the pattern of stress resistance we have documented would also be seen among a set of bird species with differential longevity, or among species of primates, or bats. There may be a multiplicity of pathways that enable evolutionary adaptation to niches that support slow aging, or there may be only a few ways to make a long-lived mammal. Learning how Nature creates a long-lived species is a key problem in biogerontology. Our data suggest that studies of the pathways that mediate inter-species differences in fibroblasts stress resistance may help us to deconstruct this aspect of evolutionary cell biology.

Figure 4.1
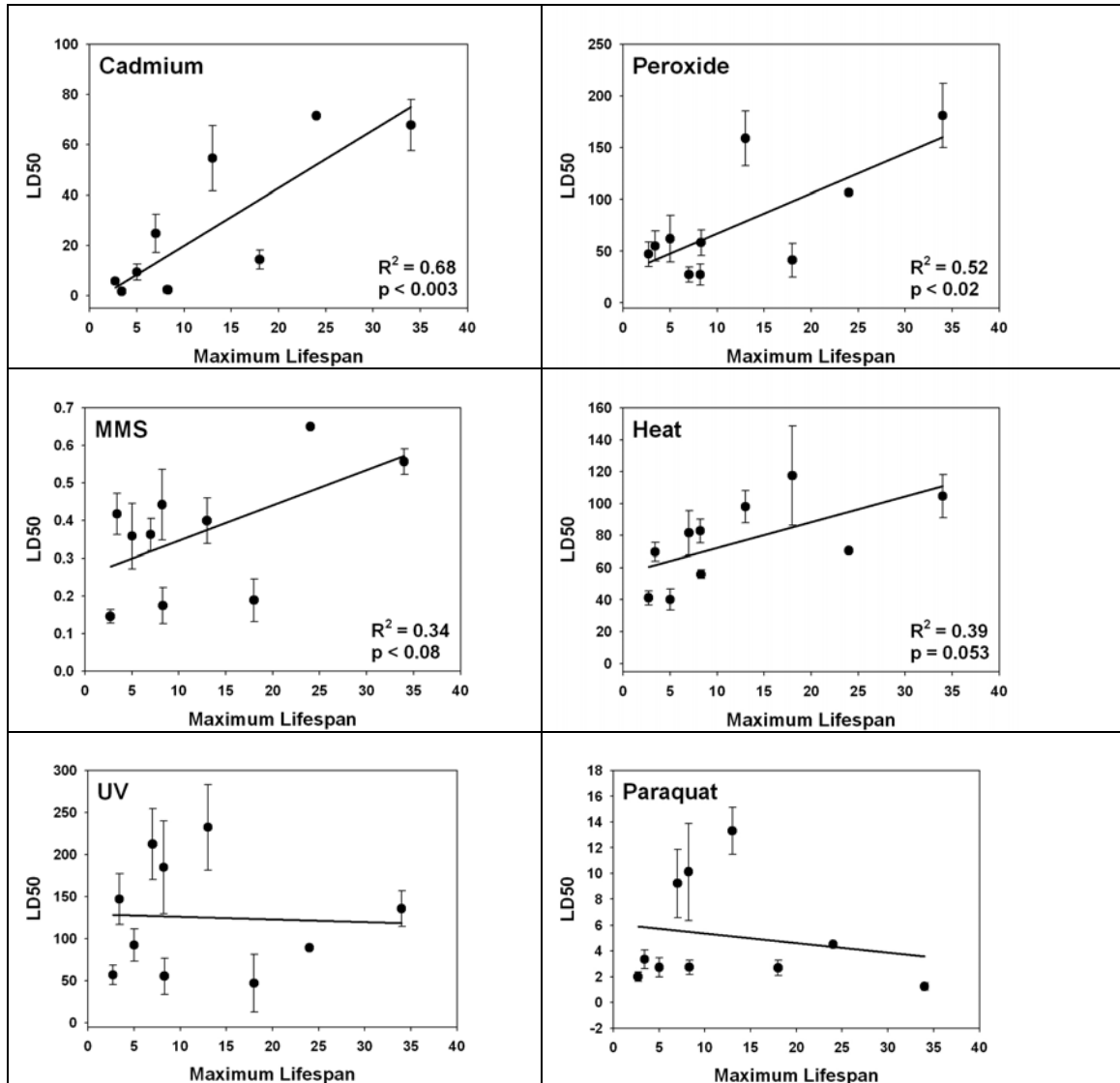
Figure 4.1. Association between maximum lifespan and LD50. Each scatterplot shows an association between species maximum life span and mean LD50 value for each of ten species (treating laboratory mice and wild-trapped mice as separate species for reasons explained in the text). From left to right, points 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 4.1**. 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 4.2**. 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 4.2
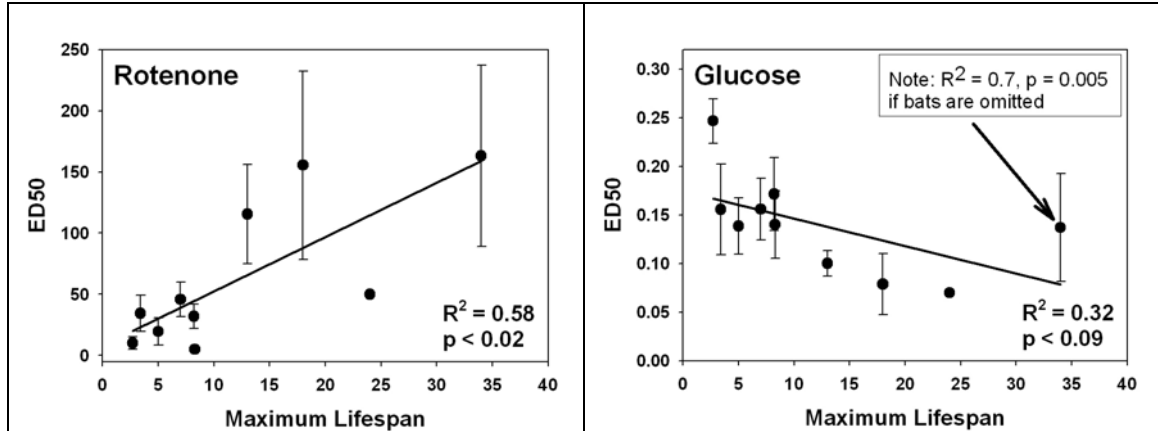
Figure 4.2 Cellular resistance to glucose deprivation and rotenone. As in Figure 4.1, except that the vertical axis shows mean "ED50" values, i.e. the dose of rotenone or glucose that led to a 50% reduction in WST-1 reduction compared to cultures in control medium, for each of ten species (treating laboratory 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 4.1: Summary of samples used in this study

Species	Common name	Life Span Estimate (yr) (a)	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 hudsonicu	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

(a) 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 4.2: 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

Table 4.3: 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**

Table 4.4: 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

Table 4.5: Comparison of cells from wild mice, laboratory mice, rats, and bats.

Treatment	Laboratory Mice	Wild Mice	Rat	Bat	p(t) – Lab vs Wild Mice	p(t) – Bat vs Wild Mice	p(t) – Bat vs Rat
Cadmium	5.7 ± 1.1	1.6 ± 1.0	9.3 ± 3.2	68 ± 10	0.00	0.00	0.00
H_2O_2	47 ± 12	55 ± 15	62 ± 23	181 ± 31	0.81	0.00	0.01
MMS	0.15 ± 0.02	0.42 ± 0.06	0.36 ± 0.09	0.56 ± 0.03	0.00	0.06	0.03
Heat	41.2 ± 4.4	69.7 ± 5.8	40.1 ± 6.5	105 ± 13	0.00	0.02	0.00
UV	56.9 ± 11.5	147.1 ± 30.2	92 ± 19	136 ± 21	0.03	0.84	0.17
Paraquat	2.0 ± 0.4	3.4 ± 0.7	2.7 ± 0.7	1.24 ± 0.29	0.26	0.03	0.08
Rotenone	10.0 ± 5.2	34.5 ± 14.8	19.6 ± 11.3	163 ± 74	0.07	0.02	0.01
Glucose	0.247 ± 0.02	0.156 ± 0.05	0.14 ± 0.03	0.14 ± 0.06	0.03	0.61	0.52

Values are mean LD50 values ± sem for N = 9 laboratory mice, N = 10 wild-trapped mice, and N = 7 or 8 bats. Significance levels show two-sided t-tests; because the distributions were non-normal, values were log-transformed prior to the t-test calculations. Units are as indicated in the legend to Figures 4.1 and 4.2. Note that high values represent increased resistance, except for glucose, in which a low value indicates resistance to glucose withdrawal.

Figure 4.S1.
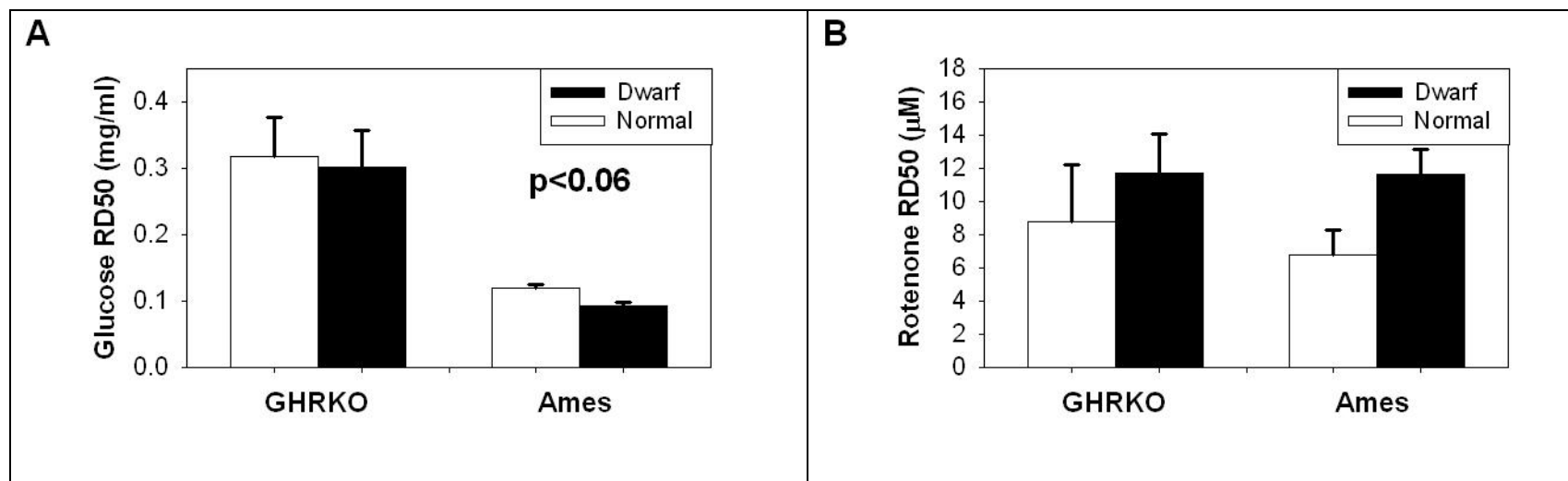
Figure 4.S1. Ames and GHRKO mouse cellular resistance to glucose deprivation and rotenone. Each bar shows the average metabolic resistance of fibroblasts from Ames dwarf mice or GHRKO mice, together with their respective controls. Panel A shows the resistance of cells from GHRKO (n=12 pairs) mice and controls and Ames dwarf mice (n=6) and controls to low glucose inhibition of WST-1 reduction. P values are from paired t-tests. Panel B shows cells isolated from both Ames (n=5) and GHRKO (n=4) mice and controls in their resistance to the metabolic inhibition of rotenone.
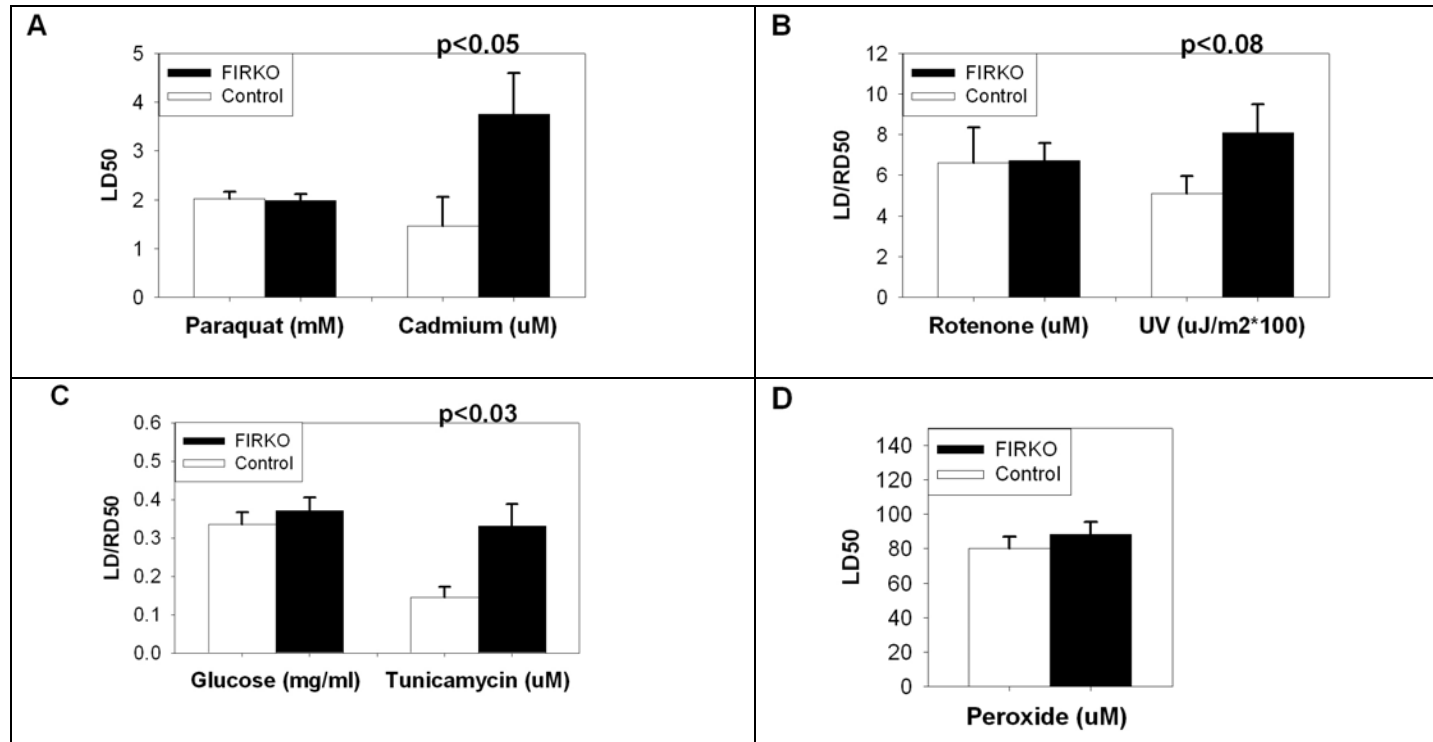
Figure 4.S2.

Figure 4.S2. FIRKO mouse cellular metabolic and cytotoxic stress resistance.

Each bar shows the average resistance of fibroblasts from the FIRKO mice and their controls (n=6). Panel A shows the LD50 of cells exposed to paraquat and cadmium, respectively. Panel B shows studies of resistance to rotenone and UV. Panel C shows resistance to the metabolic inhibition of low glucose and the toxic effects of the ER stressor tunicamycin. Panel D shows resistance to hydrogen peroxide, and shows no difference between FIRKO and control cells. P values were derived from unpaired t-tests. All cell lines were derived from cells obtained from Dr. Stephen Russell.

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CHAPTER V

NRF2 SIGNALING: A MECHANISM FOR CELLULAR
STRESS RESISTANCE IN LONG-LIVED MICE

Foreword

Following the encouraging results in Chapter II describing dwarf cellular resistance to culture in low glucose and exposure to rotenone, the less encouraging results in Chapter III showing few differences in dwarf cellular metabolism and mitochondria, and the more encouraging results in other species and long-lived mice in Chapter IV, Chapter V expands upon my initial work and to provides a plausible mechanism for previously observed phenomena. This chapter was aided by the previous work in this thesis and by significant published findings outside our lab during the time of the work. The combination of information from my experiments and other's led me to hypothesize that changes in WST-1 reduction were mediated by the PMRS, and furthermore, that the PMRS may be regulated by the Nrf2/ARE system. The resulting data support this hypothesis and provide several consistent, although not completely proven, mechanisms for my previous observations, as well as for previous work in our lab on cellular stress resistance. Thus, this chapter connects my previous work on "metabolic differences" with mechanisms that may involve cellular stress resistance, and represents an advance in our knowledge of the dwarf cellular system. Note: the beginning of the chapter is a manuscript, currently being submitted, but the appendix has been included to show the rest of the results relevant to these studies.

Abstract

Transcriptional regulation of the antioxidant response element (ARE) by Nrf2 is important for the cellular adaptive response to toxic insults. New data show that primary skin-derived fibroblasts from the long-lived Snell dwarf mutant mouse, previously shown to be resistant to many toxic stresses, have elevated levels of both Nrf2 and multiple Nrf2-sensitive ARE genes. Dwarf-derived fibroblasts exhibit many of the traits associated with enhanced activity of Nrf2/ARE, including higher levels of glutathione, and resistance to plasma membrane lipid peroxidation. Furthermore, treatment of control cells with arsenite, an inducer of Nrf2 activity, increases their resistance to paraquat, hydrogen peroxide, cadmium and ultraviolet light, rendering these cells as stress resistant as untreated cells from dwarf mice. Augmented activity of Nrf2 and ARE-responsive genes may coordinate many of the stress-resistance traits seen in cells from these long-lived mutant mice.

Introduction

The discovery of single gene mutations that extend lifespan, first in invertebrates (1-3) and then in mice (4-6) has provided new momentum for defining the molecular mechanisms that control the aging process. Since Harman first proposed the free radical theory of aging (7), many lines of evidence have suggested that oxidative stress plays an important role in aging. In roundworms (*C. elegans*) (8;9) and fruit flies (*D. melanogaster*) (10-12), mutations resulting in resistance to toxic stresses, both oxidative and otherwise, tend to result in increases in longevity. The relative importance of oxidation damage as a regulator of lifespan is more controversial. Longevity is often associated with resistance to oxidative injury within and among species, but most attempts to retard aging by anti-oxidant treatments have failed to show beneficial effects, and mutations that promote oxidative damage in mice have often had little impact on lifespan (13-17). Utilizing cells from the Snell dwarf mouse, a model of extended

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longevity, we are attempting to find the mechanism behind cellular stress resistance, in hopes of relating this resistance to the delayed aging of the Snell dwarf animal.

Snell dwarf mice are homozygous for a mutation at the Pit-1 locus which causes improper development of the anterior pituitary, leading to low levels of growth hormone, thyroid stimulating hormone, and prolactin in young and adult mice (18;19). These pituitary defects lead to diminished circulating levels of insulin-like growth factor 1 (IGF-1) and thyroxine, which in turn result in reduced size and hypothermia. Snell dwarf mice, like the closely related Prop1 mutant Ames dwarf (4), live approximately 40% longer than littermate controls on several different background stocks (5;20), and show delay in many forms of aging-related pathology (5;21;22).

Previous work has shown that primary dermal fibroblasts derived from the tails of young adult Snell dwarf mice are resistant to many types of cytotoxic stress (23;24), including agents that kill cells at least in part via reactive oxygen species (ROS), such as paraquat, peroxide, cadmium, and others that cause cell death through other pathways, including UV light, the DNA alkylating agent MMS, and heat. In addition, fibroblasts from dwarf mice are resistant to the non-lethal effects of culture in the presence of rotenone or at low concentrations of glucose, each of which diminishes the cells' ability to reduce an external tetrazolium dye (WST-1) (25). Relative resistance to the lethal effects of cadmium, heat, peroxide, and MMS, and to the metabolic effects of rotenone and low glucose, were also characteristic of fibroblasts from relatively long-lived rodent species, suggesting that these properties might be involved in the evolution of longevity (26). The mechanisms that underlie the metabolic and stress resistance of cells from Snell dwarf mice and species of long-lived rodents are not yet understood.

The plasma membrane redox system (PMRS) is a group of NAD(P)H reductases which serve to pass electrons into and across the plasma membrane (27). The

PMRS acts to maintain the redox status of important antioxidants such as coenzyme Q (ubiquinone), α-tocopherol, and ascorbate (27-29), which are thought to protect cells from exogenous oxidative stress, in particular by protecting membranes from lipid peroxidation chain reactions (30). The PMRS may also have a function in cellular communication via reactive oxygen species (31;32), and in maintaining cellular redox status via NAD(P)H recycling (33). The specific proteins that contribute to PMRS function are not completely characterized, but include NADH quinone oxidase 1 (NQO1), and ascorbate free radical reductase (also known as cytochrome b5 reductase), as well as several activities attributed to multiple proteins (27;34). Reduction of extracellular tetrazolium dyes, including WST-1, has been used previously as an index of integrated whole-cell PMRS activity, but the extent to which WST-1 reduction is influenced by specific plasma membrane enzyme complexes, intracellular electron donors, and extracellular electron acceptors is not fully understood (35-37).

The expression of at least one PMRS enzyme (NQO1) is under the control of a promoter known as the antioxidant response element (ARE) (38;39). The ARE, also called the EpRE (electrophile response element), is a transcriptional promoter element thought to be important for cellular adaptation to oxidative stress (40;41). The genes involved in the ARE are involved in many aspects of stress resistance, including glutathione regulation, catalase expression, cellular redox control, and proteasome function (42). The activity of ARE promoters is modulated by the NF-E2-related factor-2 (Nrf2), which was discovered in 1994 based on its ability to bind the NF-E2/AP1 repeat in the promoter of the beta-globin gene (43). Nrf2 was extensively characterized by Yamamoto, who showed that Nrf2 is a post-translationally regulated gene whose activation of the ARE is crucial for the adaptive response to electrophiles (44-47). Under normal, unstressed circumstances, Nrf2 is found mostly in the cytosol bound to kelch-like ECH-associated protein 1 (Keap1), which promotes its ubiquitination and degradation by the proteasome (48). Nrf2 can be dissociated from Keap1

indirectly through signal transduction (phosphorylation), or directly through oxidative damage to crucial cysteine residues on the Keap1 protein. After dissociation from Keap1, Nrf2 translocates to the nucleus and binds to promoters containing the ARE sequence, activating their transcription (47). There are many proteins with ARE sequences in their promoters, most of which help protect against reactive oxygen species (ROS), including enzymes involved in glutathione synthesis (49;50) and maintenance (51), protein turnover (52), antioxidant expression (53;54), oxidant inactivation (55), NADPH synthesis (56;57), toxin export (56), and prevention of inflammation (58).

Despite the well-documented relationship between Nrf2 and protection from oxidative stress, relatively little has been published evaluating the role of Nrf2 in aging. Recent studies in *C. elegans* have suggested that the Nrf2 homologue, SKN-1, is necessary for the lifespan extension seen with dietary restriction (59) and insulin pathway disruption, and overexpression of SKN-1 can increase worm lifespan (60). Further work has suggested that a Keap1 heterozygous loss of function mutation in *Drosophila melanogaster* can increase Nrf2 activity, increasing both oxidative stress resistance and lifespan in male flies only (61). Analogous studies in mice have been complicated by the lack of viability of the Keap1 knockout mouse (62), and has been mostly limited to observations of decreased Nrf2 signaling with increased age (63), and increased Nrf2 signaling with calorie restriction (64). We report here a series of studies consistent with the idea that augmented Nrf2 activity may contribute to several forms of stress resistance seen in cultured fibroblasts from long-lived Snell dwarf mice.

Methods

Animals. 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. Sires were treated with growth hormone and thyroxine for

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increased body size and fertility. Tail skin biopsies were taken from 3–6 month-old mice.

Primary and secondary cell cultures. Tail skin biopsies (3-5mm) were obtained and cultured as previously described. Briefly, skin samples were washed, diced, and digested overnight in 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 (Atlanta Biological, Lawrenceville, GA), antibiotics, and fungizone (complete media) at 37° with 5% CO_2 in air. After collagenase treatment, cells were dislodged, centrifuged and resuspended in complete media. Approximately 2.5×10^5 cells in 3 ml media were seeded into tissue culture flasks of 25 cm^2 surface area and called passage 0. Cells were fed after 3 days (2/3 volume media replacement), and split by trypsinization after 7 days into 75 or 175 cm^2 flasks at a density of 1×10^4 cells/cm^2. Each passage was split at a 7-day interval, with approximately 2/3 total volume of media replaced at day three. Cells used in the assays described were confluently plated at ~10^5 cells/cm^2 in the third passage.

Metabolites. Cells for metabolic studies were plated confluently in 60mm dishes. Following overnight incubation, cells were washed three times in PBS and treated in parallel with various metabolic stresses for 2 hours. They were then washed in ice-cold PBS and lysed with either trichloroacetic acid (TCA) or sodium hydroxide (NaOH). Lysates were centrifuged to remove protein and neutralized to ~pH 6.5 before testing. Lysates were then separated and quantified on an HPLC column as previously described (65;66).

Plasma membrane purification. Plasma membranes were purified by the method of Navas et al. (67). Briefly, cells were trypsinized as described above, and plated confluently (~6 x 10^6 cells) in 150 mm Falcon® tissue culture dishes (Becton-Dickinson). After allowing the cells to adhere overnight, the medium was removed and the cells were washed with ice-cold PBS, scraped into 14 ml tubes,

and spun down at 4° for 5 minutes at 1000 X g. PBS was removed, followed by a 10 minute incubation in swelling buffer (10 mM HEPES-KOH, 15 mM KCl, 1.5 mM Mg acetate, 1 mM DTT, 1 mM PMSF). Cells were then broken in a dounce homogenizer for approximately 2 minutes, followed by addition of concentrated swelling buffer (80 mM HEPES-KOH, 150 mM KCl, 8.5 mM Mg acetate, 1 mM DTT, 1 mM PMSF). Homogenates were spun down for 10 minutes at 100 x g to remove unbroken cells and nuclei, followed by a 1 hour centrifugation at 40,000 x g. Pellets were resuspended in water and subjected to aqueous two-phase partition (6.6% dextranT500 (Pharmacosmos, Holbaek, Denmark), 6.6% polyethylene glycol (Integra, Renton, WA), 0.25 sucrose, 5 mM potassium phosphate, pH 7.2). Phases were mixed by inversion, followed by centrifugation at 750 x g, and the upper phase was transferred to a centrifuge tube and spun down for 1 hour at 40,000 x g. Pellets were resuspended in water, flash frozen in liquid nitrogen, and stored at -80° for enzymatic assays. Plasma membrane purity was measured as previously described, (68) using antibodies (Santa Cruz Biotechnology) specific for the proteins of the plasma membrane (anti-Na+/K+-ATPase a-subunit), endoplasmic reticulum (anti-Ribophorin I), and mitochondria (anti-cytochrome c oxidase subunit I), using immunoblotting techniques described below.

PMRS enzymatic assays.   PMRS enzymatic assays were performed as previously described on 10 μg of purified plasma membrane protein.   For cytochrome c reductase (69), sample was added to a final solution containing 50 mM Tris, pH 7.6, 0.2 mM NADH, 0.1%Triton-X-100, and 20 μmol/l cytochrome c. For NADH CoQ reductase activity (69), enzyme sample was added to a solution containing 50 mM Tris, pH 7.6, 0.2 mM NADH, 0.1%Triton-X-100, and 0.2 mmol/l coenzyme Q0.   NADH-AFR reductase (68), absorbance at 340 nm was measured kinetically after addition of 66 x 10^{-3} units of ascorbate oxidase in solution containing 50 mM Tris-HCl, pH 7.6, 0.2 mM NADH, 0.1% Triton X-100, and 0.4 mM fresh ascorbate and 20 μg of plasma membrane. NQO1 (68) (DT diaphorase) activity was measured at 550 nm in solution containing 50 mM Tris-

HCl, pH 7.6, 0.2 mM NADPH, 0.1% Triton X-100, and 10 μM menadione, 75 μM cytochrome c, with or without 10 μM dicoumarol. NQO1 activity was calculated as the difference between the uninhibited sample and the dicoumarol inhibited sample.

Lipid peroxidation measurement by DPPP+ fluorescence. Cells were measured for lipid peroxidation as previously described (70). Briefly, cells were plated confluently (~10^6 cells per well) on 6-well plates and allowed to adhere overnight. Cells were then washed and placed in either complete or serum-free medium containing 2% BSA for ~24 hours. The medium was removed, the cells were washed with PBS, and the various media (in either complete or DMEM) were added in a solution containing 50μM DPPP (Invitrogen). Media shown are complete (DMEM+10% serum), serum-free (DMEM only), complete with stress (50 μM hydrogen peroxide or 40 μM rotenone), glucose free media (10% serum) and DMEM with stress (50 μM hydrogen peroxide, or 10 μM cadmium chloride). Cells were incubated at 37°C in the dark for 30 minutes, after which they were washed twice with PBS, scraped in 125μl PBS, and 100μl of cell solution were added to a 96 well plate. The plate was read for DPPP fluorescence (with excitation at 340nm and emission at 405nm) on a Spectramax 96 well fluorescence plate reader. The remaining 25μl of solution was tested for protein content.

ROS production. ROS production was measured by the change in fluorescence of the mitochondrial superoxide sensing dye MITOSOX (Invitrogen) and the cytosolic superoxide sensing dye dihydroethidium (DHE) (Invitrogen). Briefly, cells plately confluently in 6-well plates were exposed to 24 hours serum deprivation, 3 hours rotenone (40 μM with serum), or no stress. MITOSOX was added at 4 μM and DHE was added at 3 μM for 30 minutes each, before washing cells in ice-cold PBS and scraping into tubes. 100μl of cells were measured for fluorescence at 485nm excitation and 590nm emission for MITOSOX and both

518nm excitation, 605nm emission and 355nm excitation, 420nm emission for DHE. The remaining cells were tested for protein content.

Immunoblots. Lysates for western blots were prepared in RIPA buffer. Lysates were aliquotted and stored at -80° until the date of use. All blots were run on SDS-PAGE gels containing 10% acrylamide (BioRad). All primary and secondary antibodies for Nrf2, Nrf1 and Keap1 were obtained from Santa Cruz Biotechnology. Secondary antibodies were conjugated with alkaline phosphatase, and blots were read after enhanced chemifluorescence (ECF, Amersham) addition on a Storm 840 fluorescence scanner. Quantification was performed using ImageQuant software.

Nrf2 mediated increase in WST-1 reduction. Cells plated confluently in 96 well plates were washed in PBS and exposed to low doses of Nrf2 activators, arsenite and tBHQ. After 24 hour incubations with these activators, WST-1 was added as previously described; cells were incubated for 3 hours and read on a 96-well plate reader.

RTPCR. Cells plated confluently in 6-well plates were treated with either 24 hours serum deprivation, 24 hours low level arsenite exposure, or no treatment (fresh media). After 24 hours, RNA was prepared using the RNeasy kit (Qiagen) following the manufacturer's instructions. RNA was frozen at -80° and stored until used for RT-PCR assays. RT-PCR was performed using the SYBR green RT-PCR kit and Quantitect primers (Qiagen) as described by the manufacturer, using a Corbett Rotor-Gene 3000 thermal cycler.

Reduced thiol (glutathione) measurement by monochlorobimane fluorescence. Cells were treated as in lipid peroxidation measurements, except that cells were incubated for 2 hours before dye (monochlorobimane, Invitrogen) was added to the cells, and were read at an excitation of 380 nm and an emission 465 nm.

<u>Total glutathione measurement.</u> Total glutathione was measured using a total glutathione measurement kit (Cayman Chemicals). The principle of the kit is an enzymatic cycling reaction where GSH reacts with 5,5'-dithio-*bis*-2-(nitrobenzoic acid) (DTNB, Ellman's reagent), producing a yellow colored acid (TNB). The mixed disulfide produced by the reaction is reduced by glutathione reductase to GSH and produces more TNB. The rate of color (TNB) production is proportional to the the concentration of total glutathione. Briefly, cells were prepared and scraped as above, followed by manual homogenization for 30 seconds with a Pellet Pestle® (Fisher). Homogenates were centrifuged at 10,000 x g for 15 minutes followed by incubation on ice prior to testing. 50μl of sample were added to 150μl of glutathione cocktail (enzymes, cofactors, Ellman's reagent), and the samples were read on a 96-well spectrophotometer at 405 nm every 2 minutes for 30 minutes with constant shaking. Results were compared to control curves for GSSG standard to obtain glutathione values.

<u>Arsenite Survival.</u> Cells were plated confluently (3X10^6 cells per well) in 96-well plates and allowed to adhere overnight. Media was removed, cells were washed with PBS, media (DMEM, 20% FCS, pen/strep, fungizone) containing varying concentrations of inhibitors were added, and cells were incubated for 1 hour at 37° and 5% CO2 before addition of 5 μl of WST-1 (Roche). After 3 hour incubations with WST-1, plates were read at 585nm on a 96-well spectrophotometer to obtain inhibition results. After reading, inhibitory media was removed, cells were washed in PBS, and returned to standard media. After a 24 hour recovery period cells were tested again with WST-1 to measure survival. Results were verified by measuring thimidine uptake as described previously (23).

<u>Stress assays with/without glucose, rotenone and arsenite.</u> Stress assays were performed as previously described (24), with slight modifications. Briefly, cells were plated confluently on 96-well plates, allowed to adhere overnight, and either serum starved for 24 hours, glucose starved 24 hours (glucose free testing), left

in complete media (rotenone testing), or serum starved with the addition of 5 μM arsenite. Rotenone assays were dosed with 5 μM rotenone 2 hours prior to peroxide addition. Except for UV assays, cells were dosed with stressor for 6 hours, after which cells were washed with PBS and returned to previous media without glucose, rotenone, or arsenite stress. UV plates were washed with warm PBS and placed in PBS while dosed with UV using a Stratolinker irradiation system set to the desired dosage, after which they were returned to serum-free (recovery) media. Survival was measured the following day by WST-1 reduction.

Protein content measurement. Protein content was measured by coomassie blue (Bradford assay, BioRad), as described by the manufacturer, except where detergent was used in cell lysis. Where detergent was present, the DC protein assay (BioRad) was used as described by the manufacturer.

Statistical analysis. Statistical analyses were performed using paired t-tests (two tailed) for comparison between dwarf and control cells, as each dwarf and control cell line pair was tested together. Graphs show the mean of combined experiments and error bars represent the standard error of the mean. Differences between treatments were calculated using repeated measures ANOVA. For calculation of LD50s, mean survival was calculated at each dose and the LD50 was calculated using probit analysis using NCSS software (NCSS, Kaysville, UT).

Reagents. Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, St. Louis, MO.

Results

Dwarf and control NAD(P)(H) responds similarly to metabolic stress

Previous data have shown that primary fibroblasts from the skin of Snell dwarf mice are resistant to the non-lethal inhibition of PMRS function induced by either glucose withdrawal or rotenone exposure, as measured by reduction of the extracellular dye WST-1 (25). Resistance to PMRS inhibition is correlated, in cells from normal mice, with the level of resistance to the lethal effects of hydrogen peroxide and cadmium, and is also seen in cells from long-lived rodent species (25;26). Because the enzymes responsible for WST-1 reduction require NAD(P)H, and because both glucose deprivation and rotenone exposure affect NAD(P)H levels, we evaluated the redox status of NAD(P)H in dwarf and control fibroblasts under control and stressed conditions (Figure 5.1). As expected, NADH levels were reduced in low glucose media and increased by rotenone, but there were no significant differences between the dwarf and littermate controls in any of the conditions tested. Similarly, there were no differences between dwarf and control cells in levels of NADPH or in the ratio of NAD(P) to NAD(P)H in any condition. These data suggest that differences between dwarf and normal cells in the ability to reduce WST-1 are unlikely to be due to differences in levels of NAD(P) metabolites, but do not rule out the possibility that differences in specific cellular compartments (e.g. cytosolic) in NAD(P)H could be important for exogenous dye reduction by dwarf cells under metabolic stress.

Dwarf cells differ from controls in PMRS activity

Having ruled out NAD(P)H redox status as the mechanism for resistance of dwarf cells to inhibitors of PMRS function, we next examined the enzymes that contribute to PMRS function per se. To do this, we prepared plasma membranes from dwarf and control fibroblasts, and tested for *in vitro* activity levels of CoQ reductase, ascorbate free radical (AFR) reductase, cytochrome c reductase, and NQ01 (Figure 5.2). We noted a significant increase in CoQ and AFR reductase activity in membranes prepared from dwarf cells. There was no difference in levels of cytochrome c reductase. Consistent with prior studies (71;72), we found very little membrane-associated NQ01, although this activity was readily

detected, at equal levels, in whole cell lysates from control and dwarf cells (not shown). The data on CoQ and AFR reductases show that some elements of the PMRS may be more active in plasma membranes from dwarf-derived cells.

PMRS protection from lipid peroxidation and cell death

The PMRS is thought to be important for protection against plasma membrane lipid peroxidation elicited by exogenous or endogenous oxidative stress. Using a dye (diphenyl-1-pyrenylphosphine, or DPPP) that intercalates into the membrane and reacts with lipid hydroperoxides, we examined the lipid peroxide formation of normal and dwarf fibroblasts in baseline and stressed conditions. As shown in Figure 5.2, there were no differences between dwarf and normal cells in DPPP fluorescence in unstressed cells, cultured with or without serum. H_2O_2, tert-butyl hydroperoxide, and cadmium each led to an increase in lipid peroxidation, which was significantly higher in control than in dwarf cells. Low glucose culture led to a significant increase in lipid peroxidation only in normal cells, and had no effect on dwarf cells. In separate experiments (Supplemental Figure 5.1), we evaluated intracellular production of ROS using MITOSOX and DHE as indicators (73), and found no difference between normal and dwarf cells in medium with or without serum, or in the presence of 40 μM rotenone. The relative resistance of stressed dwarf fibroblasts to lipid peroxidation is consistent with the data suggesting higher levels of PMRS function in the plasma membranes of these cells.

If the PMRS helps protect cells against plasma membrane oxidation damage, inhibition of the PMRS should sensitize cells to the lethal effects of H_2O_2. Consistent with this idea, Figure 5.2 shows that exposure to rotenone, or culture in low glucose medium, can each lead to a significant decrease in LD50 to H_2O_2. The suggestion that elevated PMRS may contribute to resistance to lethal oxidants is consistent with our previous work (25) showing (a) that individual mice whose cells are relatively resistant to the inhibition of PMRS by glucose withdrawal are also resistant to the lethal effects of H_2O_2 and cadmium, and (b)

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that resistance to the lethal effects of these two agents accompanies the resistance to PMRS inhibition noted in fibroblasts from long-lived rodent species (26).

Nrf2 activators increase PMRS activity

The increase in PMRS activity could in itself account for resistance of dwarf cells to the metabolic effects of rotenone or low glucose medium, and contribute to some forms of cytotoxic stress noted in the dwarf cells. We next wished to see if broader changes in signal transduction could account for increases in PMRS activity and simultaneously to a broader spectrum of stress resistance pathways. We focused on the antioxidant response element (ARE), which has been reported to regulate expression of at least one PMRS enzyme (NQO1) (74). To test if the ARE could affect the PMRS activity involved in WST-1 reduction by skin-derived fibroblasts, we exposed cells to low levels of ARE activators arsenite and tert-butyl hydroquinone (tBHQ). The results (Figure 5.S2) showed that ARE activation increased WST-1 by reduction 20-60%, depending on the cell line, consistent with a role for ARE induction in increasing PMRS mediated WST-1 reduction.

Dwarf Cells Have Higher Steady State Levels of Nrf2

To test if the increased PMRS activity in dwarf cells might be due to an increase in ARE activation, we measured the steady-state levels of the transcription factor, Nrf2, a key mediator of ARE activation. The results (Figure 5.3) show that both in normal unstressed conditions and after the addition of the Nrf2 activator sodium arsenite, dwarf cells have ~25-35% greater Nrf2 protein levels than cells from littermate controls. This result was statistically significant ($p < 0.01$), and there were no differences in the amount of activation by arsenite. Since the degradation of Nrf2 is regulated by its binding partner Keap1, we also tested to

see if Keap1 was down-regulated in dwarf cells, but we found no differences between genotypes or conditions in steady-state Keap1 levels, or in the levels of the related transcription factor, Nrf1, with or without arsenite treatment.

Increased Nrf2-dependent transcription in dwarf fibroblasts

To test whether the relatively small increase in Nrf2 levels in dwarf cells might affect ARE-responsive genes, we used quantitative RT-PCR to measure mRNA levels of six ARE genes, as well as of Nrf2 itself. The results (Figure 5.4) show that Nrf2 transcription levels do not differ between dwarf or controls cells, with or without exposure to 5 μM arsenite. In contrast, there are robust and statistically significant differences between dwarf and control cells in expression of each of the ARE-related genes tested. Four of the genes show approximately two-fold increased expression in dwarf cells prior to arsenite exposure. These include glutamate cysteine ligase modifier subunit 1 (GCLM1), an important rate-limiting enzyme of glutathione synthesis (42;75;76); heme-oxygenase 1 (53;77); metallothionein 1, an important enzyme for metal-binding (78;79); and thioredoxin reductase, an important enzyme for ascorbate recycling and protection from oxidative stress (49;80-82). Two others, NADH quinone oxidoreductase 1 (NQO1), and glutathione S-transferase A1 (GSTA1), showed no genotype differences in the control condition, but were higher in dwarf cells after arsenite treatment. All six genes respond vigorously to arsenite treatment, as expected. The mRNA levels in the arsenite-treated cells are significantly higher in dwarf cells than in control cells for five of the six genes (the exception is GSTA1, where $p < 0.1$). Thus many Nrf2-responsive genes are expressed at elevated levels in dwarf derived cells, prior to and/or after exposure to an ARE activator.

Increased glutathione and resistance to arsenite in dwarf cells

Because ARE induction is known to cause increases in reduced glutathione (83) we also measured levels of total reduced thiols (as a surrogate for reduced glutathione; Figure 5.5A) as well as total glutathione content (Figure 5.5B) in dwarf and normal cells under various conditions. Under baseline conditions, dwarf cells had significantly higher levels of both reduced and total glutathione, compared to control cells. As expected, arsenite (5 μM for 24 hours) produced a significant increase in both reduced and total glutathione; thus both measures remained higher in dwarf than in normal cells after arsenite exposure. Because arsenite is known to damage glutathione sulfhydryl groups, we also tested if dwarf cells were resistant to the toxicity of arsenite. The results (Figure 5.5C-D) show that dwarf cells have a robust, four-fold higher resistance to the toxic effects of arsenite, perhaps due to their increased glutathione and other ARE-related stress defenses. These increases in glutathione levels and arsenite resistance suggest that ARE upregulation could function to protect dwarf cells from some toxic stresses.

Nrf2 activation increases cytotoxic stress resistance

The hypothesis that elevated Nrf2 and ARE function contribute to the stress resistance of cells from dwarf mice implies that induction of ARE responses in normal cells should increase their resistance to lethal injury. We found (Fig. 5.6) that exposure of normal cells to arsenite does indeed increase their resistance to multiple cellular stresses, including oxidative stressors peroxide, paraquat, and cadmium, as well as to UV irradiation. Arsenite treatment of cells from dwarf mice (not shown) also produced increases in resistance to multiple forms of lethal stress, but the effect was less dramatic than the response of controls cells shown in Figure 5.6; responses of arsenite-treated dwarf cells were thus similar to those of arsenite-treated control cells, consistent with the hypothesis that higher Nrf2 signaling may contribute to the resistance of dwarf fibroblasts to multiple forms of lethal stress.

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Discussion

Our previous work (23-25) has shown that cultured primary fibroblasts from long-lived rodent species, and fibroblasts from long-lived mutant mice, are resistant to apoptosis induced by a wide range of lethal stresses, and resistant to the inhibition of the PMRS by rotenone or by low glucose media. Here we advance and provide initial data to support the hypothesis that these properties of cells cultured from Snell dwarf mice are due to increased activity of the ARE-specific transcription factor Nrf2. We have shown that Nrf2 protein levels are higher in dwarf cells (Fig 5.3); that plasma membranes of dwarf cells have higher activity of two ARE-sensitive PMRS activities (Fig 5.2) and that these membranes are resistant to stress-induced lipid peroxidation; that incubation in rotenone or in low glucose medium, each of which inhibits PMRS function, also decreases the resistance of fibroblasts to lethal effects of H_2O_2; that four Nrf2-sensitive genes are at higher levels in dwarf than in control cells and that five Nrf2-sensitive genes are higher in dwarf than in control cells after exposure to the Nrf2 inducer arsenite; that levels of reduced and total glutathione are higher in dwarf cells prior to and after arsenite treatment; and that arsenite increases resistance of control cells to paraquat, hydrogen peroxide, cadmium and ultraviolet light, rendering these cells as stress resistant as untreated cells from dwarf mice. Although no one of these findings provides definitive evidence that the unusual properties of Snell dwarf fibroblasts are caused by higher Nrf2 levels, they are all fully consistent with this idea, and provide a plausible link to connect alterations in resistance to lethal stresses to the resistance of dwarf cells (and, potentially, cells of long-lived species) to inhibition of PMRS activity.

Increased Nrf2 transcriptional activity has previously been suggested to contribute to the effects of caloric restriction (CR), another model for increased longevity in rodents, by showing increased antioxidant response element (ARE) transcription in the livers of calorie restricted mice (64). This report also used an Nrf2 knockout strain to suggest that Nrf2 was necessary for the anti-carcinogenic

effects of CR. There was no evidence that Nrf2 was required for increased lifespan in response to CR, although interpretation was complicated by the short lifespan of the Nrf2 knockout mice on the control (ad libitum) diet. It is possible that the increase in lifespan of the Nrf2 knockout under CR was due to decreased production of reactive oxygen species (84;85), which would not be affected by Nrf2. Because no Nrf2 positive controls were included in the study, it is unknown whether the effects of CR simply restore Nrf2 knockouts to a normal lifespan, or increase lifespan similarly to a normal, Nrf2 positive mouse on CR.

Information about the possible role of Nrf2 in control of aging and lifespan in intact organisms is still quite limited, but provocative. In *C. elegans,* the Nrf2 homologue SKN-1 has been found to be required for diet-restriction induced longevity (59). SKN1 was also reported to be necessary for life extension by disruption of insulin-like signaling pathways, and overexpression of SKN-1 led to an increase in lifespan (86). In flies, heterozygous deletion of Keap1, which increases Nrf2 activity, increases oxidative stress resistance and lifespan in males (61). In mice, data suggest that Nrf2 knockouts are phenotypically normal in terms of in growth and development (87), but highly susceptible to a variety of oxidative stresses, including acetominaphen toxicity, hyperoxic lung injury, pulmonary fibrosis, glucose induced oxidative cardiomyocyte damage, colitis-associated colorectal cancer, and many others (reviewed in (42)). Based on the study mentioned above (64) and another (88), Nrf2 knockout mice appear relatively short-lived due to development of immune mediated hemolytic anemia, but large formal studies have yet to be published. It would be of interest to evaluate lifespan of mice engineered to overexpress Nrf2. Knockout mice with diminished expression of Keap1 do show increased Nrf2 activity, but these mice die at an early age due to hyperkeratinosis of the esophagus and forestomach (62), and thus evaluation of lifespan in mice with elevated Nrf2 function may require development of systems for conditional overexpression of Nrf2 or conditional deletion of Keap1.

Our new data suggest a mechanism for the relative resistance of cells from dwarf mice to the inhibitory effects of glucose on PMRS: dwarf cells have both higher levels of PMRS function in their plasma membranes, and also higher levels of reduced thiols (mostly reduced glutathione) in both standard and low glucose media (Figure 5.5). Our work also shows that Nrf2 activation acts to increase WST-1 reduction, suggesting that an increase in electron donation from glutathione and parallel increase in PMRS expression may be sufficient to account for the increased resistance of PMRS to glucose shortage. We hypothesize that under low glucose conditions, the increased level of available reduced glutathione makes up for the lack of NADH, preserving the ability of dwarf-derived fibroblasts to reduce exogenous dyes like WST-1. Our data do not offer a clear explanation for the resistance of dwarf cells to rotenone, but we hypothesize that rotenone may affect the PMRS directly to prevent WST-1 reduction, and that differential expression of PMRS enzymes partly compensates for this inhibition in dwarf cells. This hypothesis is supported by previous work showing that rotenone binds to NADH oxidoreductases (89-91) other than those associated with Complex I of the mitochondrial electron transport chain, and by our own data (not shown) suggesting rotenone can affect PMRS activity in isolated plasma membranes.

Variations in activity of the plasma membrane redox system (PMRS) could also influence the pace and consequences of the aging process. Previous work has shown that PMRS antioxidants and enzymatic activity decreases with age, and that these effects are prevented by the effects of caloric restriction (27;69). The decrease in membrane antioxidant levels and changes in redox defenses could leave cells from older animals more susceptible to plasma membrane damage through lipid peroxidation. Lipid peroxidation is thought to be important in several age-related diseases, such as Parkinson's disease, Alzheimer's disease, and thyroid disease (92-94). The work described here suggests that Coenzyme Q reducing activity and ascorbate free radical reducing activity, both elements of the PMRS, are each increased in dwarf membranes. In contrast, another PMRS

enzyme, NQO1, was not increased in these cells, suggesting that augmented PMRS function in dwarf-derived cells likely reflects an increase in expression of cytochrome b5 reductase or another, less studied enzyme. It is not clear if cytochrome b5 reductase is activated by Nrf2 signaling, although the promoter region of the murine cytochrome b5 reductase 2 gene contains at least one putative ARE site. It is also possible that increased PMRS function in dwarf membranes could be due to increases in other PMRS enzymes, including elements not yet described. The increase in PMRS function, together with the increase in glutathione and glutathione peroxidase (95), provide a plausible explanation for the protection of dwarf fibroblasts from lipid peroxidation. Inferences about the possible effects of increased PMRS function on redox homeostasis and cell growth and signaling (reviewed in (34;96)) are at this point purely speculative.

Augmented Nrf2 signals, which could lead to increased levels of reduced and total glutathione, may also account for the resistance of dwarf-derived fibroblasts to lethal oxidative damage. Previous work has shown that there is a shift towards oxidation in glutathione redox state in the brain, liver, heart, kidney, eye and testis of aged mice (97), perhaps leading to a more oxidative environment and increased susceptibility to oxidative damage in tissues from aging animals (98). Glutathione is the most prevalent of the redox molecules in the cell, and an increase of 25% as seen in dwarf cells could prevent the age-related shift towards oxidation in cellular redox state, thus lowering cumulative levels of oxidative damage (97). Furthermore, increased glutathione synthesis extends lifespan in *Drosophila,* showing that glutathione may have a direct role in the aging process (76). Not only does increased glutathione provide the ability to increase activity of key antioxidants such as glutathione peroxidase, but the change in cellular redox status creates a more reducing intracellular environment helping to buffer oxidative stress.

We have previous noted, in a study of genetically heterogeneous mice (25), that those animals whose cells are resistant to the effects of glucose levels of PMRS function are also relatively resistant to the lethal effects of peroxide and cadmium. Our new data suggest a mechanism for this correlation: increased expression of ARE-related genes and the PMRS. Augmented transcription of ARE-regulated genes, approximating a two-fold change in most of the genes evaluated (Fig 5.4), may lead to increased expression of glutathione and induction of phase II detoxification genes and thus improve resistance of dwarf cells to lethal stresses. The ~25% increase in cellular glutathione may also help to protect these cells from oxidative stress, and is consistent with previous work showing that glutamate cysteine ligase (GCL) levels, glutathione-S-transferase (GST) activity and GSH levels were markedly increased in the livers of 3, 12 and 24 month-old Ames dwarf mice (99). Both GCL and GST are regulated by the antioxidant response element, and further study of ARE-mediated gene expression in vivo will be informative.

Our data are consistent with the idea that increased Nrf2 expression in cells from dwarf mice enhances their resistance to cytotoxic and metabolic stresses. In this context, it will be valuable to learn more about how differentiation in the specific endocrine environment of the dwarf mouse alters Nrf2 expression, and how these cellular traits are maintained through several passages in culture. It is also unclear how increased Nrf2 levels in dwarf cells preferentially activate some ARE genes instead of others. We found that both NQO1 and GSTA1 mRNA levels did not differ in dwarf cells, while the transcription of four other ARE genes (HO-1, GCLM1, MT1, and TXRD) were approximately two-fold higher in dwarf cells. These differential effects could involve phosphorylation, acetylation, or other modifications that may affect Nrf2 binding to specific ARE sites. Current work on Nrf2 has focused on largely on factors that regulate its stabilization and nuclear transport, but much less is known about selective activation of the 200 or more ARE-regulated genes.

Analysis of expression of Nrf2-sensitive genes in tissues of Snell dwarf mice, and mice of other long-lived stocks, may provide insights into the paths by which altered production of pituitary hormones renders these mice resistant to multiple late-life diseases and degenerative changes. Previous work on Nrf2 has suggested that PI3K, Akt, PKC, and MAPK may be directly or indirectly important for Nrf2 activation (100-105). PI3K and Akt are known to be directly downstream of growth factor and insulin-like signaling, and changes in the expression or activity of either kinase due to lack of signaling during development could result in wide ranging changes including increased Nrf2 activity. Figure 5.7 summarizes this model, in which altered hormonal signals, modulating Nrf2/Keap1 interaction and/or Nrf2 binding to ARE sites, alter multiple aspects of cellular defense, including improved DNA repair (24;106), resistance to lethal stresses, maintenance of reduced thiol levels, and augmentation of PMRS function. Documentation and clarification of these postulated steps may shed light on the cellular basis of disease resistance and longevity in pituitary dwarf mice.
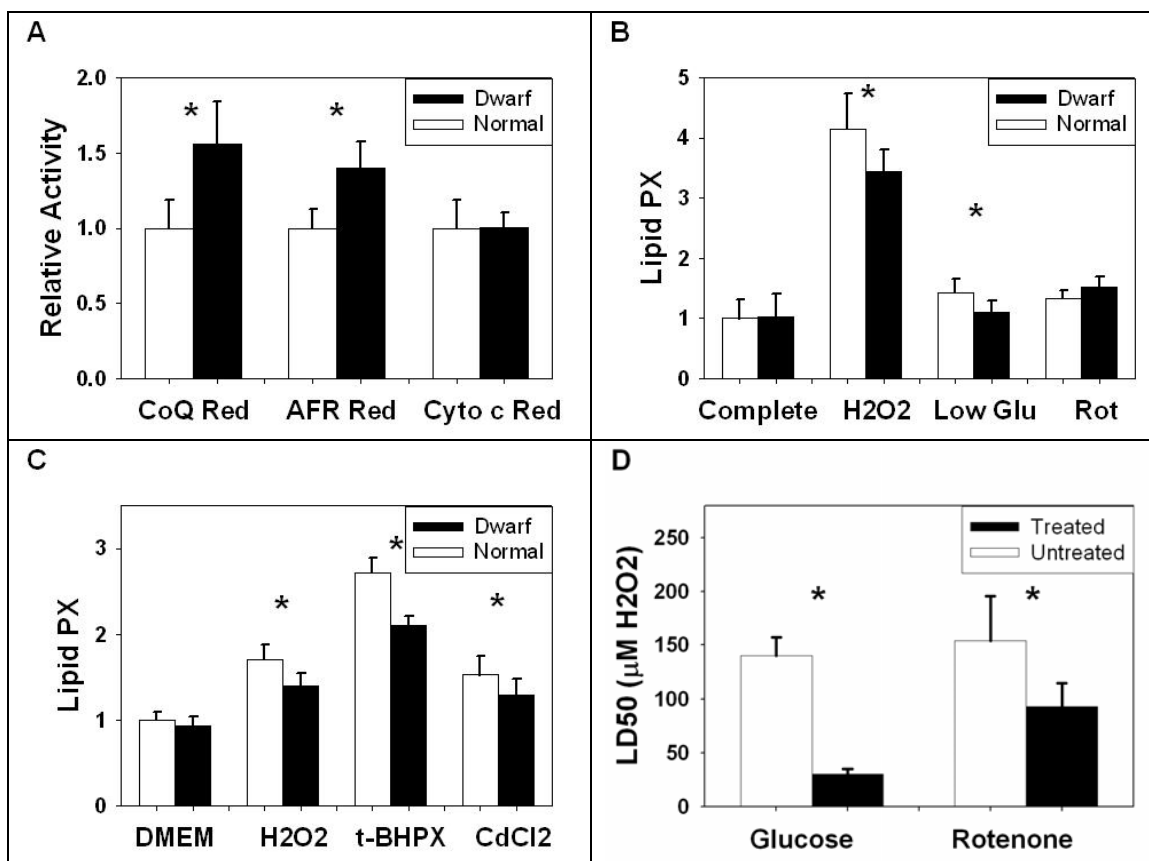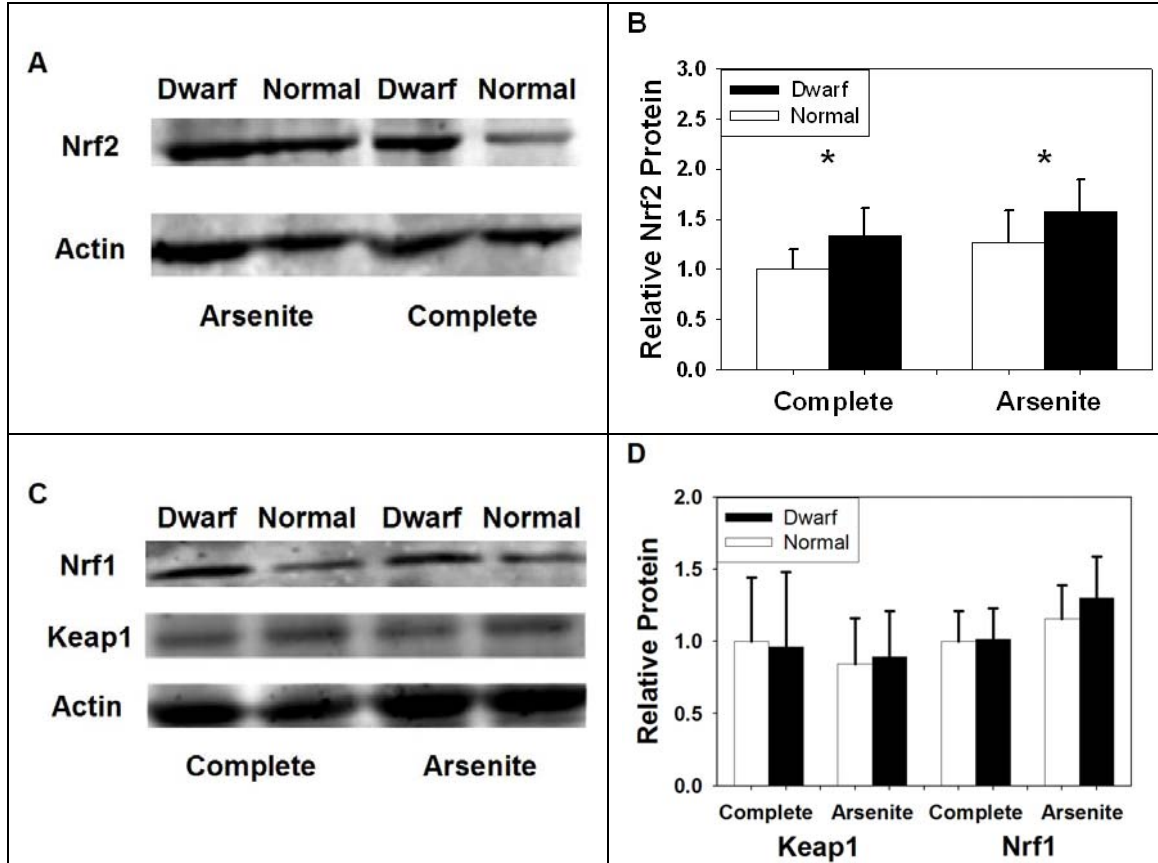
Figure 5.1.
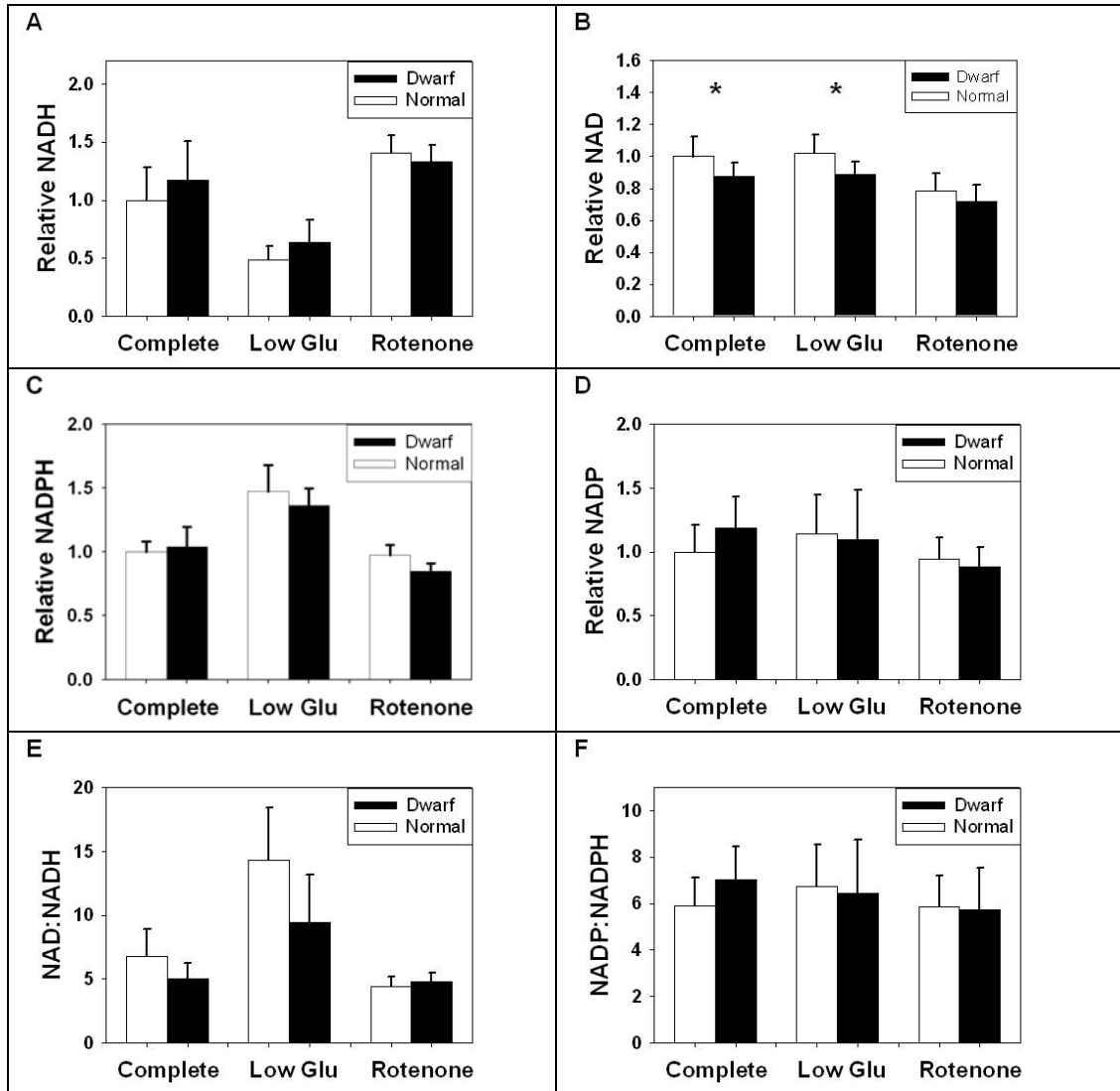
Figure 5.1. **Measurement of the cellular redox status of nicotinamide derived molecules.** Figure 5.1 shows the levels of NAD and related molecules, measured by HPLC, using the culture conditions that are typically used for assessment of PMRS function. Panel A shows the levels of NADH, measured after alkaline extraction, of dwarf and control cells that had been cultured in control (complete) medium, low glucose medium (0.25 mg/ml glucose, 20% dialyzed serum), and rotenone containing medium (high glucose, $10\ \mu M$ rotenone). Panel B shows the same conditions measuring NAD in a parallel acid extraction. Panels C and D were measured similarly to A and B, only measuring NADPH and NADP. Finally, panels E and F show the ratios between the redox molecules, showing clear effects of culture condition, but no differences between dwarf and control cells. Error bars = SEM, n=12, * denotes p<0.05 by paired t-test.
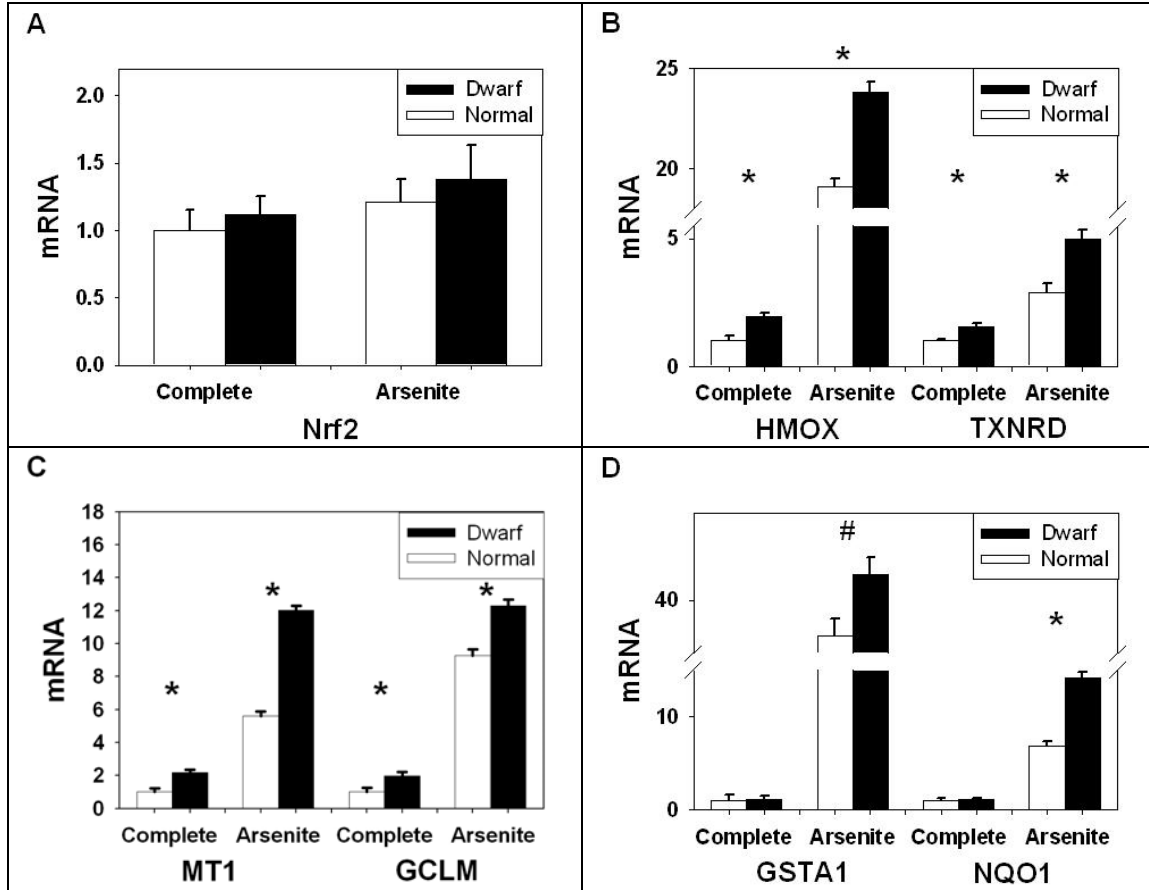
Figure 5.2.

Figure 5.2.   PMRS function and lipid peroxidation.   Panel A shows the catalytic function of PMRS enzymes measured in purified membranes from dwarf and control fibroblasts.   Dwarf membranes had significantly higher ($p<0.01$) levels of coenzyme Q reductase and ascorbate free-radical reductase activities, but there were no differences in cytochrome c reductase.   Panels B and C show levels of lipid peroxidation with (3B) or without (3C) serum.   Dwarf and control cells have equally low levels of lipid peroxidation in control conditions.   However, after treatment with 50 μM H_2O_2, 50 μM t-butyl hydroperoxide, 10 μM cadmium, or 0.25 mg/ml glucose, dwarf cells had significantly lower levels of lipid peroxidation measured by the fluorescent dye, DPPP ($p<0.05$, n=12).   t-butyl hydroperoxide has a measurable ability to oxidize DPPP alone; these background values were measured and subtracted from each sample.   Panel D shows the LD50 for H_2O_2 of normal cells, either untreated, or exposed to low glucose media (0.2 mg/ml) or rotenone (10 μM).   Error bars = SEM.

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Figure 5.3.

Figure 5.3. Nrf2 protein levels are increased in cells from dwarf mice. Panel A: representative immunoblot for Nrf2 in fibroblasts from normal and dwarf mice. Panel B: quantification of results from n = 12 blots from control cells or those preincubated in the presence of arsenite (10 μM for 4 hours). (*) indicates statistical significance by paired t-test at $p < 0.05$. Panels C and D: immunblot assays for Keap1 (n=4) and Nrf1 (n=5). Error bars = SEM.
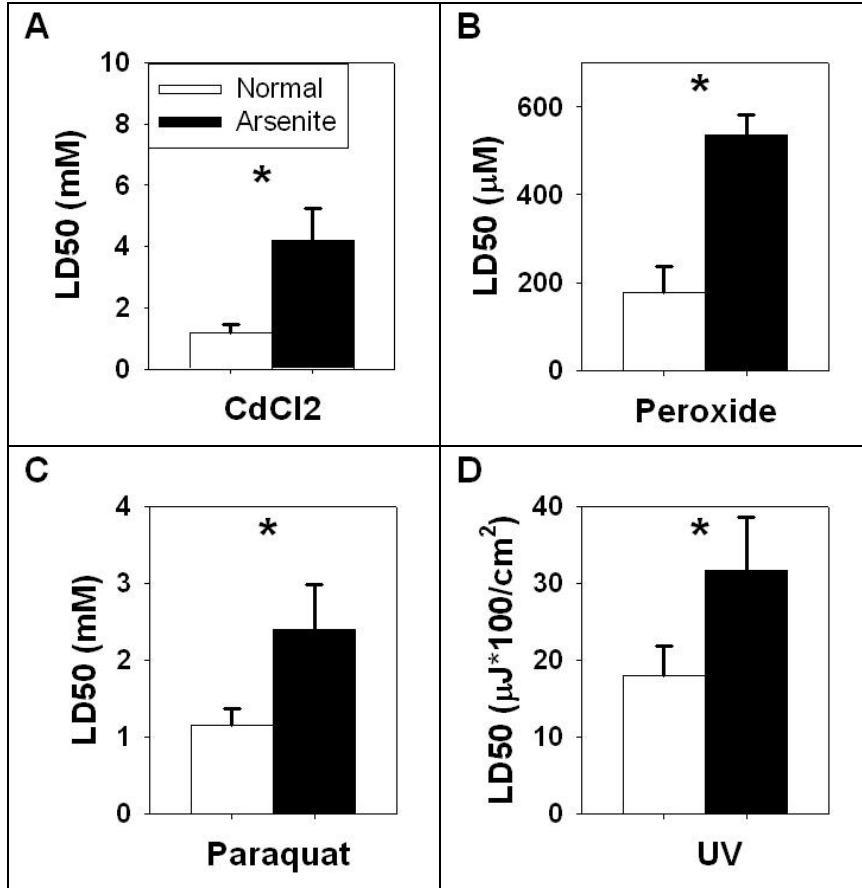
Figure 5.4.

Figure 5.4. Fibroblasts from dwarf mice have high levels of several ARE-dependent mRNAs. Each panel shows transcriptional levels of RNA derived from dwarf and control cells in complete medium and after arsenite treatment (10 μM for 24 hours). N = 6 pairs of cells were evaluated for each transcript. (*) indicates statistical significance at $p < 0.05$ by paired t-test; (#) indicates $p < 0.1$ for GSTA. Error bars = SEM.
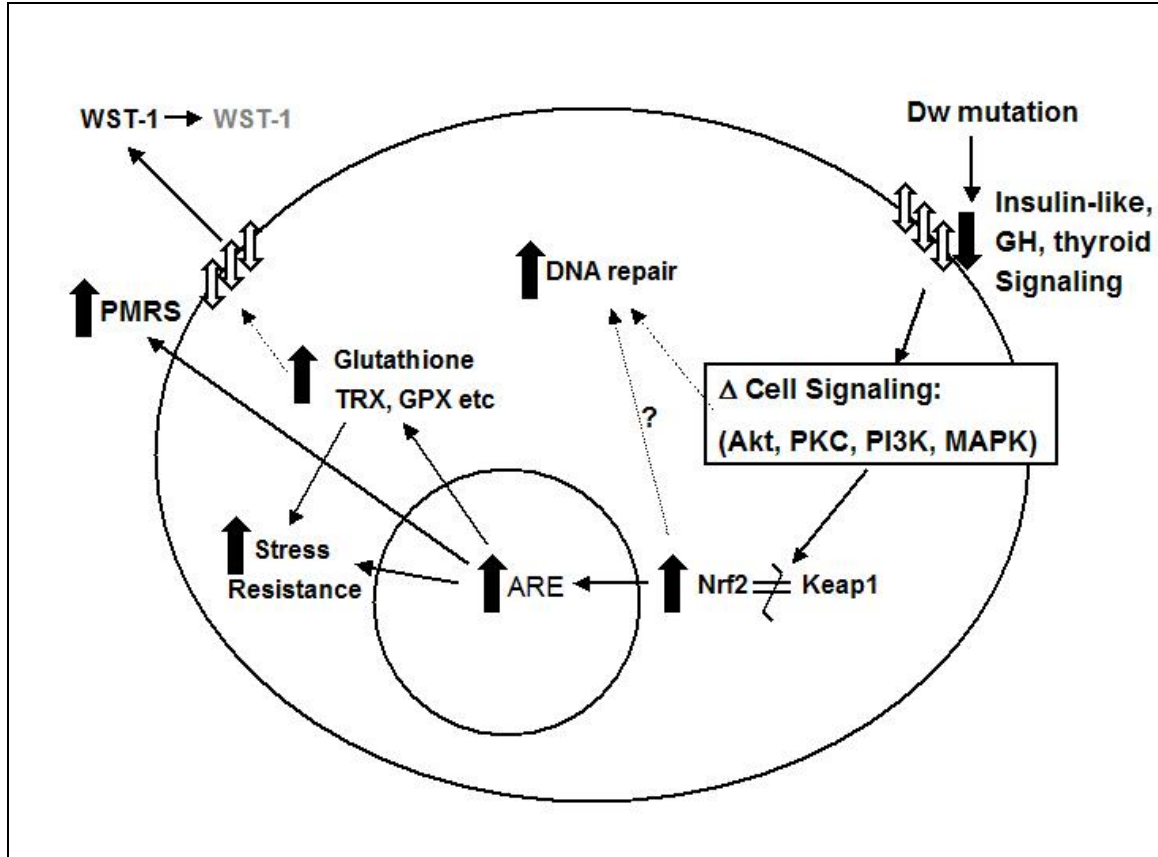
Figure 5.5.
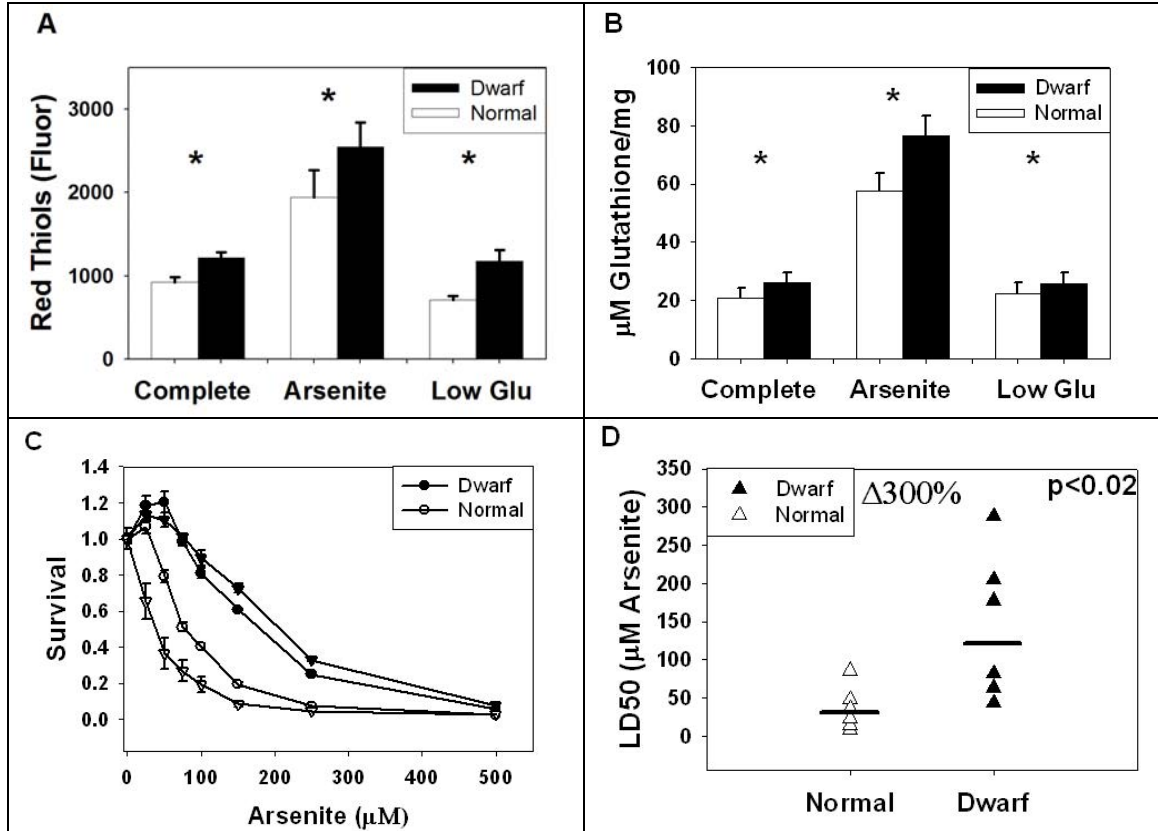
Figure 5.5:   Dwarf cells have increased glutathione levels and resist arsenite toxicity.   Panel A: levels of total reduced thiols in arbitrary units in control cells and those exposed to arsenite (10 μM for 24 hours), or low glucose (0.25 mg/ml for 2 hours).   (*) indicates statistical significance (p < 0.02) by paired t-test, n = 8 pairs.   Panel B: total glutathione content in each of the indicated conditions.   Panel C: cell survival at various arsenite concentrations; the figure shows results for two individual control (open) and two dwarf mice (filled symbols).   Panel D: compilation of LD50 results from experiments like that shown in Panel C, for n = 6 pairs of mice; each symbol represents a different donor mouse.   Error bars = SEM.

Figure 5.6.

Figure 5.6. Augmentation of stress resistance of normal cells by arsenite pre-treatment. Each panel shows LD50 values for cells exposed to the oxidative stressors cadmium, peroxide, and paraquat, or the non-oxidative stress of UV irradiation (UV-C), either in control cells from normal (non-dwarf) mice, or after 24 hr pre-incubation with 10 μM arsenite. (*) indicate $p < 0.05$ by paired t-test for N = 6 pairs. Error bars = SEM
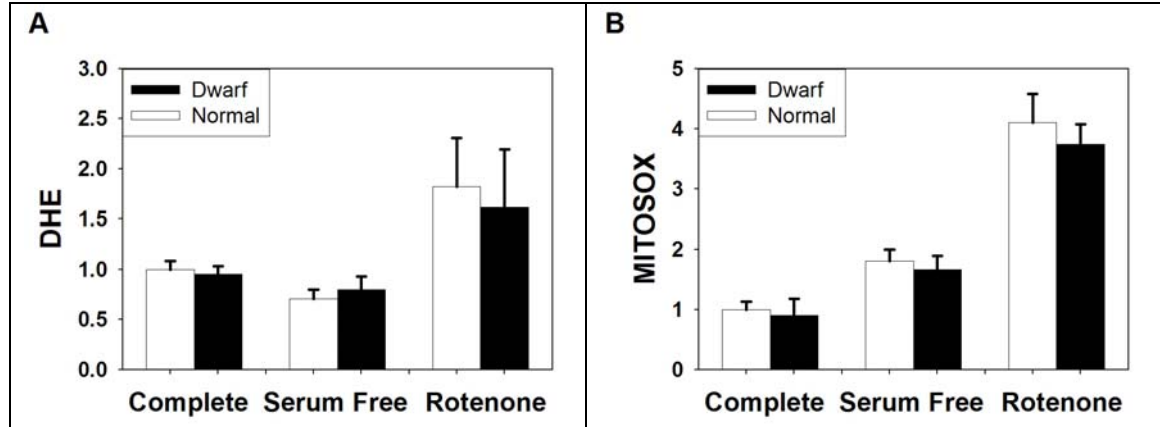
Figure 5.7.

Figure 5.7. Model of dwarf cellular resistance to toxic and metabolic stress.
A speculative model of differences between dwarf and normal cells in various aspects of cellular function; upward-pointing arrows indicate augmentation in cells from dwarf mice compared to control cells. The model postulates that hormonal differences in the dwarf mice initiate and maintain stable epigenetic changes, perhaps through PI3K, Akt, PKC or MAPK, all of which are known to affect Nrf2 levels or function. These changes act to modify Nrf2, releasing it from Keap1 and promoting activation of ARE genes. Downstream of Nrf2 activation, proteins important for glutathione synthesis and reduction, peroxide detoxification, and the PMRS, combine to increase cellular resistance to oxidative stresses, such as peroxide, cadmium, arsenite, paraquat, and to the nonoxidative stress of UV irradiation. Furthermore, the increase in PMRS activity, and in glutathione electron donation, allows these cells to resist the non-toxic effects of metabolic stresses such as glucose deprivation and rotenone exposure.

Figure 5.S1.

Figure 5.S1.   Dwarf and control cells do not differ in ROS production. Figure 5.S1 shows the relative fluorescence of two dyes that measure cellular production of superoxide, DHE and MITOSOX.   The results (n=4) show that dwarf and control cells have equal production of superoxide in complete media, in serum free media, and after exposure to rotenone (40 μM for 4 hours). Rotenone treatment increased the production of superoxide in both cell lines, but the effect did not differ between genotypes.

Figure 5.S2.
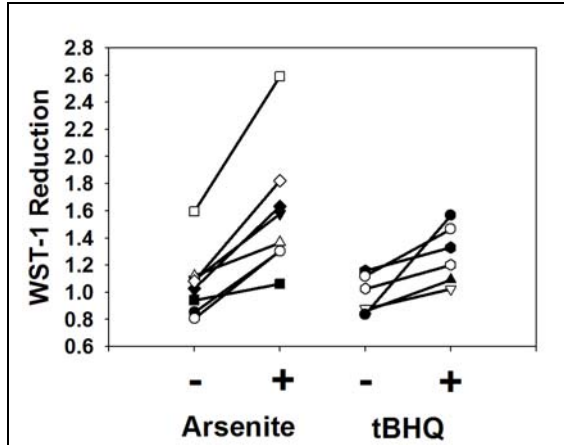
Figure 5.S2. Effects of Nrf2 activators on WST-1 reduction. Figure 5.S2 documents the effects of 24 hours of Nrf2 activation by arsenite (3 μM) or tBHQ (5 μM) treatment on dwarf and control cells; these agents induce 20-80% increase in WST-1 reduction over untreated controls. Each symbol represents a different cell line tested with and without activation, and is connected by a line to the same cell line after activation. Neither treatment showed differences in the response by dwarfs and controls (not shown).

Appendix

The main section of chapter V will be published as a manuscript, but does not include the entire data set for work performed regarding the PMRS. For the sake of completeness, figures from four additional PMRS or Nrf2 related data sets are included here. The rationale for this work is described in chapters I and V, as these data add further insight into the previous observations.

Figure 5.A1 shows broad (six-log) dose curves for the effect of rotenone on WST-1 reduction. Each line in the graph represents a different control cell line. The data show that rotenone inhibits WST-1 reduction at low doses but increases WST-1 reduction at higher doses. These data suggest that rotenone has multiple effects at different concentrations. It seems likely that the increase in WST-1 reduction at higher concentrations could represent an increase in availability of NADH caused by inhibition of Complex I of the mitochondrial electron transport chain. The diminution of WST-1 function at lower doses would, in our model, represent effects of this agent on some other cellular target, potentially the PMRS.

Figure 5.A2 shows data compiled using inhibitors for specific aspects of the PMRS. We attempted to identify other agents that might, like rotenone or culture in low glucose, inhibit WST-1 reduction without also leading to cell death, at inhibitor concentrations suggested from the literature. The results suggest that none of the PMRS inhibitors were able to completely inhibit WST-1 reduction without also causing cellular toxicity. Some inhibitors, such as the NQO1 inhibitor dicoumarol (Fig. 5.A2A), produced a nearly 50% decrease in WST-1 reduction with little cell death, whereas others, like the NAD(P)H oxidase/flavin center inhibitor diphenyleneiodonium (DPI) (Fig. 5.A2B), were toxic to cells at lower concentrations than their inhibitory dose. This result suggests that because the PMRS is a group of multiple enzymes, no specific type of PMRS inhibitor is able to completely inhibit WST-1 reduction. However, the results also

show that in most cases, dwarf cells were resistant to the toxic effects of PMRS inhibitors (Fig. 5.A2C-D). These resistances varied from ~20% to a two-fold resistance, suggesting that dwarf cells are better able to withstand the cellular stress brought on by PMRS inhibitors. Because PMRS inhibitors are not all specific for PMRS components and their mechanism of toxicity is unknown, it is difficult to prove whether dwarf cell resistance to their toxicity derives from increased PMRS expression, but the results suggest that dwarf cells are resistant to toxicity from many PMRS inhibitors.

Figure 5.A3 shows data testing the hypothesis that rotenone may affect the PMRS directly. Using the isolated plasma membranes described in this chapter, we tested whether the addition of rotenone ($10\mu M$) could inhibit the activity of the PMRS, and whether the effect was (by hypothesis) lower in cell membrances from dwarf mice. The results suggest that rotenone does affect the PMRS, but paradoxically that it increases *in vitro* activity (for three of the four tested activities) rather than inhibiting activity as we hypothesized. The data also show that control membranes were more affected in the two activities (CoQ reductase and AFR reductase) where dwarf cells were increased over controls, but that other activation/inhibition was similar (cytochrome c reductase and NQO1). Without further examination, these data are difficult to explain, but I hypothesize that if the mechanism of rotenone action at the PMRS is similar to its action at complex I, it may prevent the passing of electrons across the membrane but could promote aberrant electron leakage on the cytoplasmic side of the membrane. In an *in vitro* assay using purified membranes, the loss of membrane polarity would allow for the given substrate (e.g. CoQ) to accept electrons on either side of the membrane, and could cause rotenone to speed this reaction. However, this hypothesis has not been tested in any critical way, and thus the mechanism of the increased PMRS activity in the presence of rotenone is presently unknown. What is known is that rotenone does indeed affect the activity of purified plasma membranes, only often in the opposite direction to that originally supposed.

Figure 5.A4 is an expansion of Figure 5.6, showing the change in cellular stress resistance after Nrf2 activation by treatment with arsenite. This figure includes the data on Snell dwarf mice as well as controls, and suggests that Nrf2 activation increases stress resistance in both genotypes, with more dramatic effects in the normal cells. The effects of arsenite activation on stress resistance of dwarf cells were not significant for any of the stresses, and approached significance ($p < 0.1$) only for UV exposure. The data set is not large enough to prove that Nrf2 induction removes differences between dwarf and control mice, but suggests that the effects on stress resistance may be smaller, proportionally, in dwarf cells.

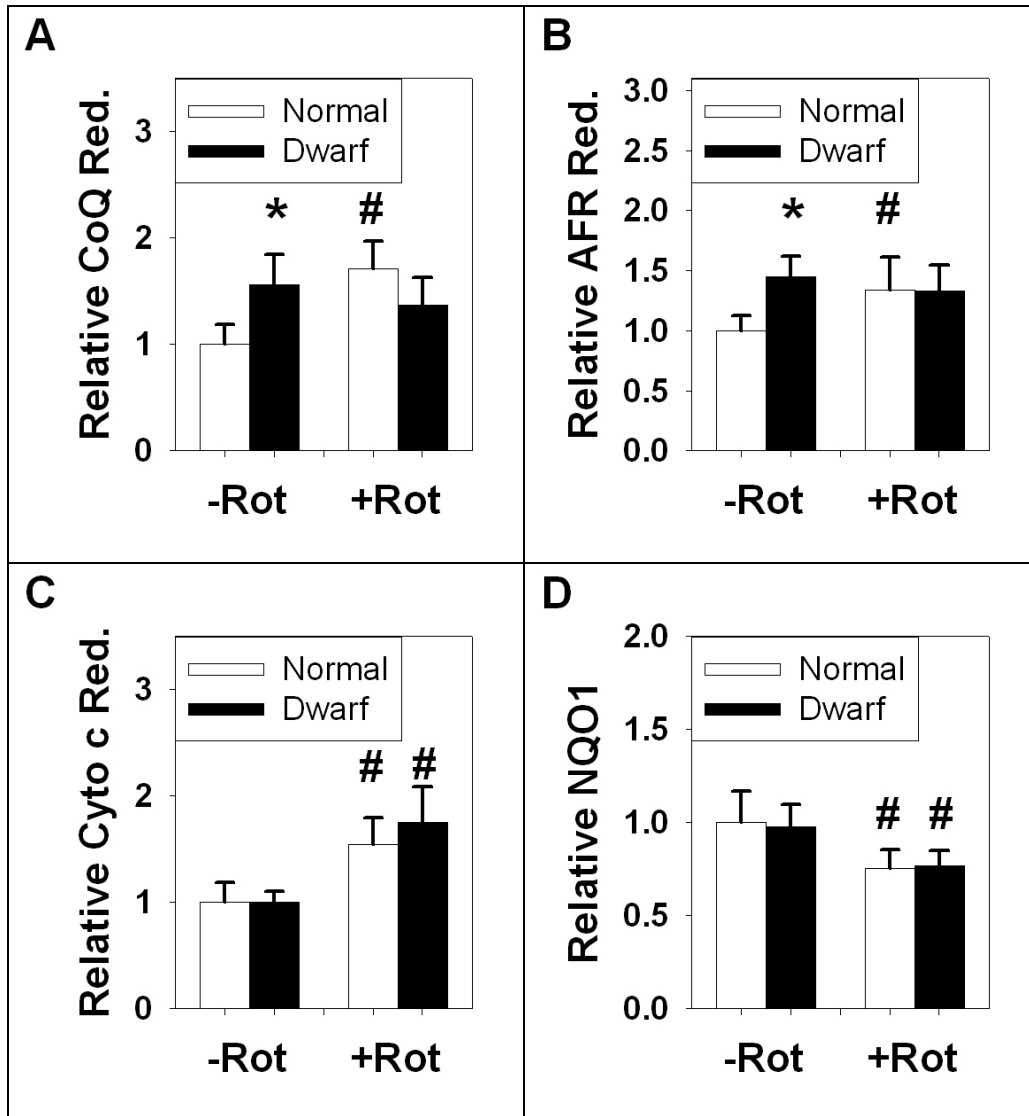**Figure 5.A1.**
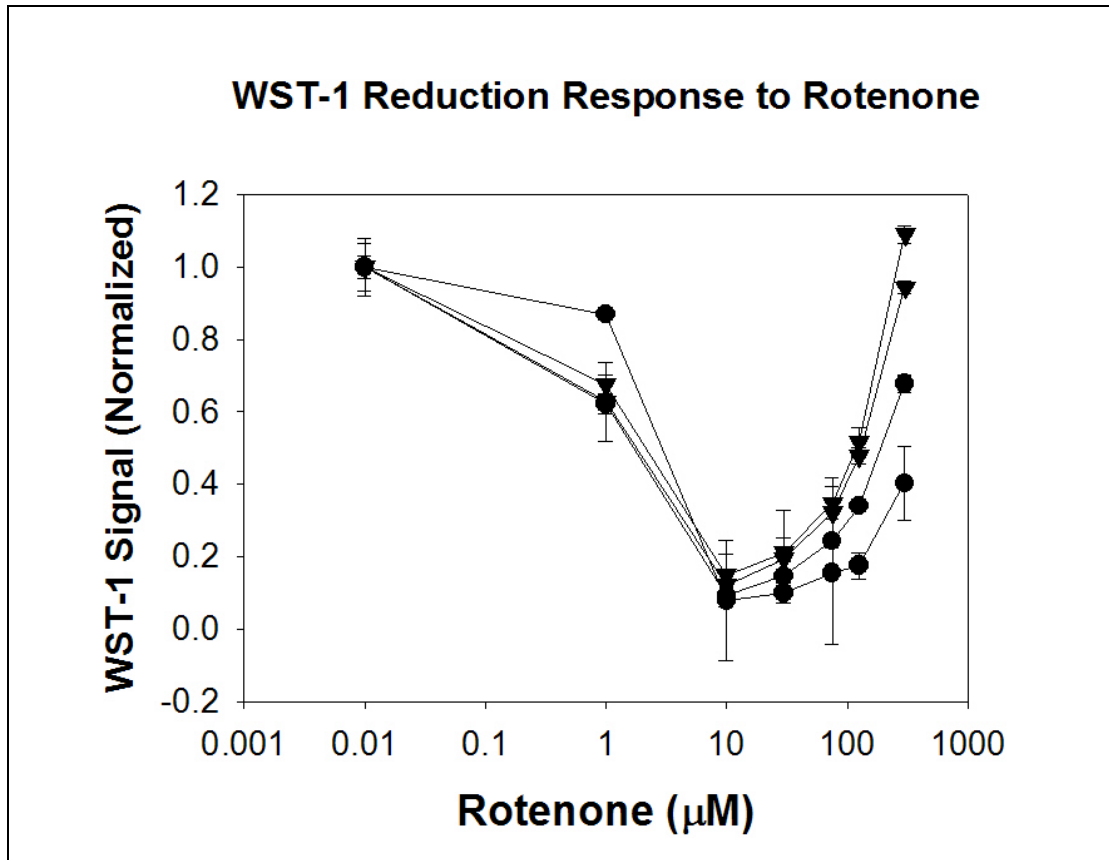
Figure 5.A1. Rotenone exposure shows bimodal WST-1 inhibition. Figure 5.A1 shows the inhibition of WST-1 reduction (3 hour incubation) in fibroblasts exposed for one hour to varying doses of rotenone in complete medium. The addition of low levels of rotenone decreases WST-1 reduction, but continued addition of rotenone reverses this effect.

Figure 5.A2.
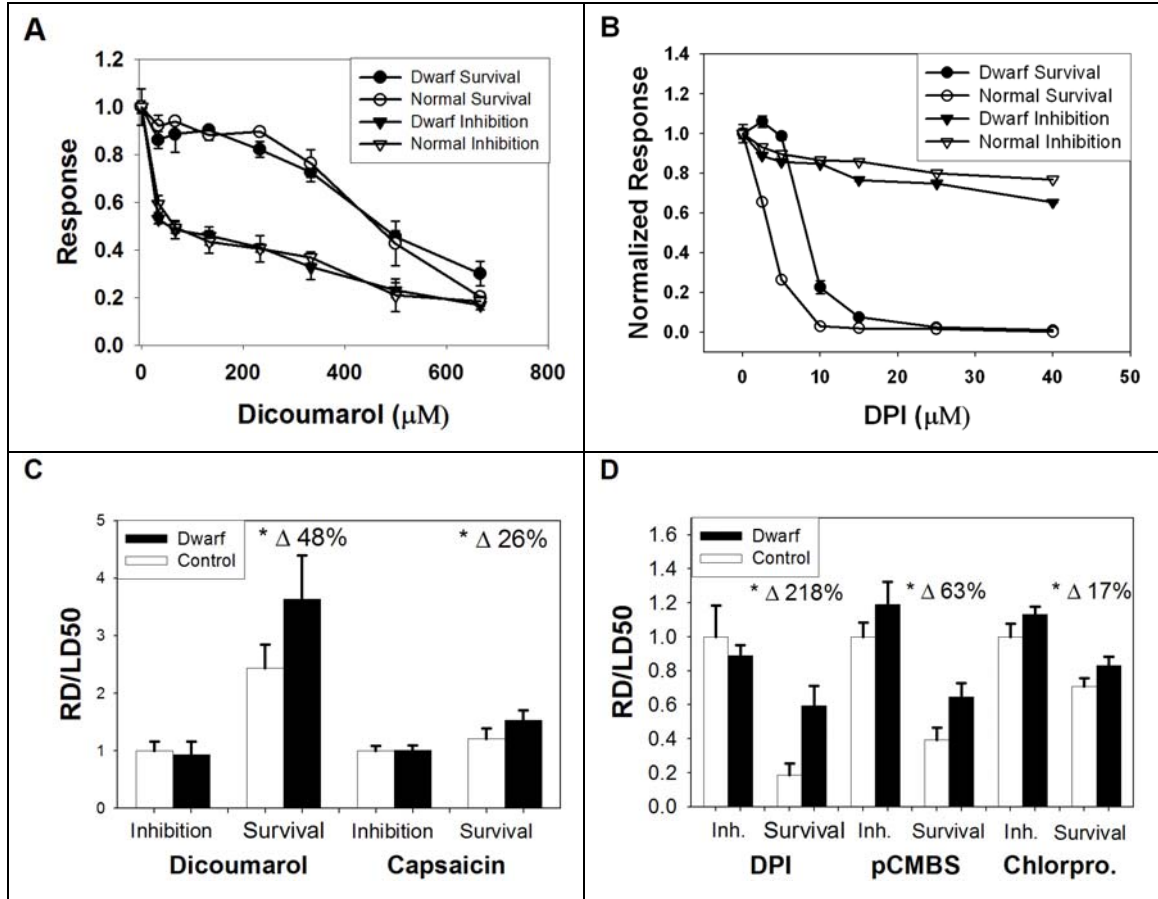
Figure 5.A2. PMRS Inhibitors only partially inhibit WST-1 reduction, but dwarf cells are resistant to inhibitor toxicity. Panels A and B show representative inhibition versus survival graphs for the NQO1 inhibitor, dicoumarol and the flavin center/NADPH oxidase inhibitor, DPI. Cells treated for 1 hour in varying doses of inhibitor were allowed to reduce WST-1 for 3 additional hours (triangles; potentially a measure of PMRS inhibition), after which they were allowed to recover in complete media overnight and were tested for survival (circles; a measure of cell death). Dicoumarol was the only inhibitor to show significant (>25%) inhibition of WST-1 signal without cell toxicity. Panels C and D show the 50% response dose and LD50 of every inhibitor used, and show that dwarf cells were resistant to the toxicity associated with all but one of the inhibitors. * denotes p<0.05 by paired t-test. N≥6.

Figure 5.A3.

Figure 5.A3. Rotenone affects the activity of the PMRS. Figure 5.A3 shows four measures of PMRS enzymatic function, with and without the addition of rotenone (20 μM). Each bar represents the mean of relative activities from multiple donors (N≥8 for each bar), and error bars represent the standard error of the mean. The (#) symbol indicates a significant difference in activity after rotenone treatment (p<0.05), and a significant difference between dwarf and control cells is denoted by the asterisk (*). For Panels A-C, purified plasma membranes were used, and for panel D, whole cell extracts were used.

Figure 5.A4.
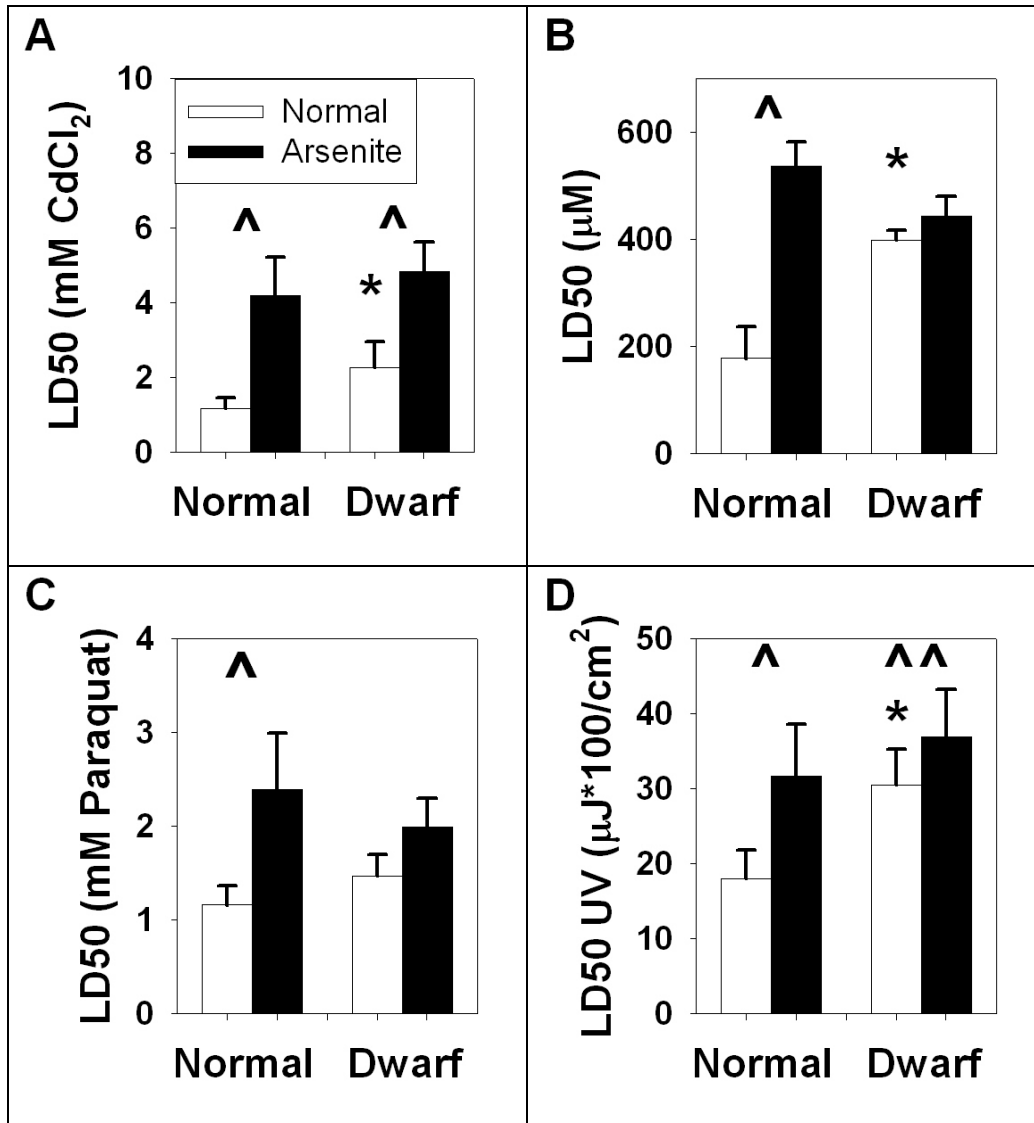
Figure 5.A4. Nrf2 activation increases dwarf cellular stress resistance to a lesser extent than controls. Figure 5.A4 shows the stress resistance of cells from normal and control dwarf mice, before (white bars) and after arsenite (5 μM for 24 hours, black bars) mediated activation of Nrf2. For each panel, a significant increase in resistance after arsenite treatment ($p<0.05$) is denoted by a (^), a nearly significant increase is denoted by (^^), and a significant difference between dwarf and control cells is denoted by (*). N=5 for each panel except for PX, where there were 5 normal cell lines and only 3 dwarf cell lines.

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CHAPTER VI

CONCLUSIONS

Summary of findings

The impetus for this work came from studies by Drs. Salmon, Murakami and Miller showing that cultured fibroblasts from long-lived Snell dwarf mice were resistant to a variety of cytotoxic stresses (*1*). Their initial finding agreed with previous work and hypotheses stating that increased stress resistance is observed with increased longevity within and between species (*2-4*). The intent of this thesis was to expand upon their data, and to provide mechanistic insight into how increased stress resistance is achieved in cells from Snell dwarf mice.

My initial hypothesis was that "glucose metabolism may be a determining factor in the survival of cells, and more specifically, may contribute to the increased cytotoxic stress resistance of cells from Snell dwarf mice." I based this on a) necessity of serum deprivation before addition of toxic insult in order to observe dwarf cellular stress resistance, and b) work showing that glucose metabolism was intricately tied to cell survival in the absence of growth factors (i.e. serum deprivation) (5-9). Thus, my first experimental approach was to test if dwarf and control cells had differential survival to culture in low glucose medium (Chapter II, III). The results showed differences between normal and dwarf cells in low glucose environments, but did not provide direct evidence about mechanisms of resistance to lethal stress, since neither dwarf nor control cells were dying in these assays. Instead, the cells were losing the ability to reduce an exogenous dye, WST-1, that we had been using as a measure of cellular viability. This initial result, that dwarf cells are resistant to the non-toxic effects of culture in low

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glucose (Chapter II), was the starting point for the rest of the work presented, and each experiment thereafter attempted to explain or expand upon this initial finding.

Chapter II documents this initial finding, in published form, as statistically significant differences between dwarf and control cells in their response to low glucose culture (10). Chapter II also includes work showing that resistance to culture in low glucose correlates with resistance to at least 2 toxic stresses, peroxide and cadmium, in genetically heterogeneous four-way cross mice. This work, performed with Dr. Salmon, suggested that metabolic differences may play a role in cytotoxic stress defense, or that both sets of cellular traits might have common control pathways, agreeing with my initial hypothesis. Furthermore, Chapter II notes that dwarf cells are resistant to the non-toxic effects of mitochondrial complex I inhibitor rotenone, in an assay similar to the low glucose assays, measuring loss of WST-1 reduction. Dwarf cellular rotenone resistance was more robust than the low glucose resistance, and helped spur further research into the differential responses to low glucose medium as measured by the external dye, WST-1.

The data presented in Chapter III document many of the pathways examined in hopes of finding a mechanism for the results in Chapter II. Because the manufacturer suggested mitochondria were responsible for WST-1 reduction and subsequent color change, and because rotenone is a mitochondrial inhibitor, we initially suspected that mitochondrial function might be important for our metabolic traits. Interestingly, work on other mitochondrial inhibitors, including other complex I inhibitors, failed to mimic the rotenone data, suggesting that rotenone inhibits WST-1 reduction through pathways that differ from simple inhibition of Complex I. Other data on mitochondrial content, membrane potential and complex activity showed that dwarf and control cells do not differ in their gross mitochondrial properties. Further work extended the lack of differences to glycolytic and other metabolic pathways, and showed that these cellular

properties did not account for how dwarf and control cells differ metabolically. The data did, however, provide information as to the mechanism of WST-1 reduction. In agreement with previous published work, we found that WST-1 reduction is extracellular and requires glycolysis, and appears to be facilitated in large part by electron donation from NAD(P)H across the plasma membrane (11-13).

While the data presented in Chapter IV were a digression from the mechanistic studies of WST-1 and dwarf cellular resistance to metabolic stress, they provided information on the relevance of glucose and rotenone responses to the biology of aging. The preliminary findings in cells from Ames dwarf and GHRKO mice supported the idea that other hormonally deficient mutant mice have cells with phenotypes similar to the Snell dwarf mouse. More importantly, the results in cells isolated from various rodent species (and the little brown bat) provided strong circumstantial evidence that cellular response to rotenone exposure and culture in low glucose evolves in concert with resistance to some cytotoxic stresses and longevity (14). Interestingly, the two strongest correlations between toxic stress resistance and longevity in Chapter IV were found using peroxide and cadmium, which were the same stresses that correlated with metabolic resistance in Chapter II. The data suggest that the non-toxic metabolic inhibitions we previously observed are not isolated to hormonally deficient mutant mice, and imply that they may be involved with, or share common control mechanisms for, toxic stress resistance. Thus, this work provided a basis for the continued search for the mechanism of dwarf cellular resistance to rotenone exposure and low glucose culture.

The data from Chapter II and III and other published work (11-13) suggested that WST-1 reduction involved the passing of electrons from NAD(P)H or other electron donors across the plasma membrane by the plasma membrane redox system (PMRS). This led to the development of a basic model for the reduction of WST-1 (Figure 6.1), where the production of reducing equivalents during

glucose metabolism provided the PMRS with electrons to pass across the membrane and eventually reduce WST-1. The model was not fully satisfactory, however, because the two metabolic inhibitions used, rotenone exposure and low glucose medium, have opposite effects on cellular NADH levels. In agreement with work in other systems and other laboratories (15-18), measuring the redox state of NAD(P)(H) (Chapter III and V) showed that low glucose decreased NADH levels and rotenone increased NADH levels, and both changes were observed equally in dwarf and control cells. A clue to the resolution of this inconsistency was the observation (Figure 5.A2) that the dose curve for rotenone inhibition of WST-1 reduction was bimodal. At high doses, rotenone increased WST-1 reduction, consistent with models in which this agent increases NADH levels by blocking mitochondrial complex I. At lower doses, in contrast, rotenone depresses WST-1 reduction, presumably through actions on one or more non-mitochondrial targets. The idea that rotenone may act somewhere besides the mitochondria is supported by data showing that rotenone can act as a promiscuous inhibitor (15). Thus, I hypothesized that rotenone may act on NADH dehydrogenase enzymes outside of the electron transport chain, perhaps including those in the PMRS.

To test this hypothesis, I examined the activities of isolated plasma membranes of dwarf and control cells and found a) that dwarf membranes have a higher activity of at least two PMRS activities, coenzyme Q reductase and ascorbate free radical reductase; and b) that rotenone changes the activity of the PMRS, albeit, in some cases, augmenting rather than diminishing PMRS enzyme activities in isolate plasma membranes. The first finding, that dwarf cells have increased PMRS activities, supports the hypothesis that PMRS levels may be involved in the higher levels of resistance, of dwarf cells, to rotenone and low glucose concentration, in that increased PMRS activity could help to overcome inhibition (rotenone) or help to continue to pass electrons when reducing equivalents are decreased (low glucose). The second finding, that rotenone increases the activity of isolated plasma membranes, is not easily explained, but

may have to do with the mechanism of rotenone action at the PMRS in membrane vesicles. As detailed in Chapter V, I speculate that the loss of membrane polarity upon cell lysis and membrane separation may cause an increase in substrate reduction because the substrate can be reduced on the interior of the membrane *in vitro*, whereas in intact cells it must be passed across the membrane. Rotenone may prevent the passing of electrons across the membrane, but in keeping with its mechanism in the mitochondria, might cause an increase in ROS production (i.e. free electrons), near the NADH binding site (cytoplasmic face of the plasma membrane). This suggestion is purely speculative, and, alternatively, it is possible that in isolated membranes the amount of rotenone used was too high to observe inhibition, and that the activation from the bimodal rotenone curve was at the PMRS. Regardless of the specific mechanism, the results with rotenone on the PMRS suggest that rotenone can affect the activity of the PMRS, in accordance with the model shown in Figure 6.1.

Finding that PMRS activity is increased in the membranes of dwarf cells could help to explain the metabolic phenomena observed in chapter II, as well as some of the data on resistance to toxic agents evaluated in previous work. For example, I found that dwarf cells exposed to stresses including hydrogen peroxide, cadmium, and media with low glucose resist the formation of lipid hydroperoxides (Chapter V), which are known to be damaging to cells (19-21).

Since differences at the plasma membrane are unlikely to explain all forms of resistance to toxic and metabolic inhibition in dwarf cells, I attempted to find the mechanism behind increased PMRS activity to see if other aspects of cellular protection might be increased. Since the literature suggested that at least one element of PMRS activity is controlled by the antioxidant response element (ARE)(22;23), I focused on the ARE and its main transcriptional regulator, Nrf2 (24;25). Initially, I found that multiple ARE activators could cause an increase in WST-1 reduction by the PMRS of 20-60%, supporting the idea that the ARE can

induce PMRS activity. This led me to test the Nrf2/ARE levels in dwarf cells, where the results (Chapter V) showed that Nrf2 steady-state levels are significantly increased in the dwarf cells, albeit slightly (30-40%), and that this increase leads to an increase in mRNA levels of ARE-responsive gene, as well as increased glutathione levels. Further data showed that activation of Nrf2 in normal cells could increase their cytotoxic stress resistance to levels even greater than that of untreated dwarf cells, and that dwarf cells have a lesser response to Nrf2 activation, supporting the hypothesis that Nrf2 signaling is responsible for many of the stress resistance phenotypes observed in dwarf mouse fibroblasts.

In summary, the results of this thesis focus on two related phenomena, resistance of dwarf cells to the inhibition of PMRS mediated WST-1 reduction caused either by the addition of rotenone or by the depletion of glucose in culture media. These two inhibitors are non-lethal, correlate with toxic stress resistance in individual cell lines, and also correlate with maximum lifespan when tested in cells isolated from several different rodent species. In examining the mechanisms behind these phenomena, gross mitochondrial or cellular metabolic differences were ruled out, and differences in Nrf2 signaling and the PMRS were discovered. Higher levels of Nrf2 seem to account for increased glutathione and prevention of lipid damage in dwarf cells, and are likely to be involved in the increased stress resistance observed in dwarf cells. These results continue the progress toward a molecular mechanism for the stress resistance of Snell dwarf fibroblasts, which is an important step toward finding how stress resistance evolves and what role it may play in increasing longevity.

Models, speculation, implications

The data in this thesis provide significant circumstantial evidence that increased stress resistance in cells from Snell dwarf mice may be caused in large part by increases in Nrf2 signaling and ARE expression. This hypothesis could explain

the observation in Chapter II, that resistance to culture in low glucose correlates with resistance to cytotoxic stresses, since increased ARE expression could explain both phenomena. Increased ARE expression could contribute to increased cytotoxic stress resistance in several ways: 1) by increasing antioxidants such as glutathione that protect cells from ROS, 2) by increasing metal binding proteins that such as metallothionein, 3) by increasing proteasomal function and cell's ability to recycle damage proteins, 4) by increasing ROS degrading proteins such as glutathione peroxidase that neutralize ROS, and 5) by increasing DNA damage sensing to enhance repair of any DNA lesions (26-31). This combination of enhancements could easily lead to the increased toxic stress resistance observed in cells from the Snell dwarf mouse. Furthermore, if Nrf2 levels are increased in cells from other long-lived species (a hypothesis not yet tested), it is possible that Nrf2 homologues in these species are upregulated in some way to provide their enhanced stress resistance.

The explanation for how increased Nrf2/ARE signaling increases Snell dwarf cellular resistance to culture in low glucose is somewhat less clear, but my hypothesis is described in Figure 6.2A. According to the work in chapter III and previously published work on WST-1 reduction (11;13;32), resistance to low glucose could have several different mechanisms, including increased cellular NADH, increased PMRS expression, or increases in alternative electron donors inside the cell. In the Snell dwarf cells, I believe that increases in alternative electron donors best explains their resistance to culture in low glucose, with the possibility that increased PMRS expression contributes as well. The data that support this hypothesis are included in Chapter V, where low glucose conditions cause a decrease in reduced glutathione (GSH) levels of normal control cells, but caused no change in GSH levels of dwarf cells. These data, along with the increased GSH observed in dwarf cells in control conditions, and published work suggesting that GSH can act as an alternative donor for the PMRS (33), are consistent with the idea that increased GSH could allow dwarf cells to better metabolize WST-1 in low glucose media. Also, because GSH is not the primary

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electron donor for WST-1 reduction, it would explain why in both dwarf and control cells' WST-1 reduction is strongly inhibited by low glucose, and increased GSH provides only a small increase (~33%) in dwarf RD50. Furthermore, my unpublished work showed that resistance to culture in low glucose and resistance to rotenone-mediated WST-1 metabolism inhibition are not strongly correlated in cells derived from heterogeneous mice (not shown), suggesting that the mechanisms for the two "metabolic inhibitions" are likely to be distinct. In support of this, rotenone exposure had no effect cellular levels of GSH in dwarf or control cells (not shown), consistent with the idea that distinct pathways lead to resistance of dwarf cells to the two tested metabolic inhibitors.

Figure 6.2 also depicts a hypothesis on rotenone's inhibition of WST-1 reduction and how dwarf cells resist this inhibition. As described partly in Chapter V, I hypothesize that at low doses rotenone can act at the membrane and inhibit the PMRS from passing electrons across the membrane to reduce WST-1. Higher doses of rotenone would cause significant inhibition of complex I of the mitochondrial electron transport chain, leading to increased cellular NADH and increased WST-1 reduction, explaining the increase on the right side of Figure 5.A2. The data from Figure 5.A3 also supports this hypothesis by showing that rotenone has an effect on the activity of purified plasma membranes; however the effect was typically stimulatory, and thus in the opposite direction than expected, as discussed in Chapter V. Together, the increase in PMRS activity found in dwarf cell purified plasma membranes (Figure 5.2) along with the lack of effect of rotenone on these PMRS enzymatic activities are consistent with the idea that increased dwarf cellular PMRS expression and/or decreased susceptibility of the dwarf PMRS to rotenone contribute to the resistance of dwarf cells to rotenone induced WST-1 metabolism inhibition. Interestingly, while Chapter V hypothesizes that increased Nrf2/ARE activity may be involved in the increased PMRS activity, the most well studied PMRS member that is also a member of the ARE, NQO1, was not increased in transcription or activity in dwarf cells (Chapter V). Because it is the best described PMRS enzyme known to

reduce AFR and CoQ, and because the other well described PMRS enzyme (NQO1) does not differ between dwarf and control cells, cytochrome b5 reductase (Cb5R) is a likely candidate to cause the PMRS activity differences observed. Cb5R has not yet been shown to be regulated by the ARE, and the mechanism of its increased expression, if it indeed underlies the increased activity in dwarf cells, is unknown. Regardless of the mechanism behind increased PMRS activity, the increased activity could account for the dwarf cellular resistance to rotenone (Chapter II), and contribute to the dwarf cellular resistance to lipid peroxidation under stress, as documented in Chapter V.

Chapter I describes how previous work in the lab by Drs. Murakami and Salmon found that dwarf cells were resistant to a variety of cytotoxic stresses. My initial hypothesis involving metabolic differences in these cells stemmed from their data that dwarf cytotoxic stress resistances required a serum deprivation step to observe. This was because serum deprivation causes a decline in glucose uptake and slows glucose metabolism (8;9). In agreement with this, dwarf cells respond better to low glucose environments as described in Chapter II. In contrast to this, the data in Chapter V describe differences in dwarf cellular Nrf2 levels and ARE expression in complete, control medium, and while I have gathered limited data suggesting that Nrf2/ARE are also increased in serum free culture (not shown), the differences appear stable after removal of serum. This begs the question, why is serum deprivation necessary for observing dwarf cytotoxic stress resistance? The answer to this question is unproven, but I hypothesize that it involves the growth and survival signals found in serum. More specifically, I hypothesize that dwarf cells are more protected from cellular damage in both control and serum free conditions, only that in control, high serum (20%) conditions, the survival factors in serum prevent many control cells from undergoing programmed cell death and thereby blunt the differences dwarf and control cells in damage accrued. When serum is not present, the damage caused by stressors will more easily cause apoptosis in both cell types, as evidenced by their decreased LD50s (1), but the decreased damage in dwarf

cells in part due to Nrf2/ARE activation will prevent some of the damage and allow for the stress resistance in dwarf cells. This theory is unproven at this time, but is supported by data on lipid peroxidation (Chapter V), where dwarf cells were resistant to lipid peroxidation in both control and serum free conditions equally. It is also supported by the data on Nrf2 and its activation in control conditions, and by the lack of necessity for a serum deprivation in non-toxic, metabolic inhibitions like low glucose culture and rotenone exposure. Finally, the constitutive and epigenetically stable increase in Nrf2/ARE could help to explain observations by another member of our lab, Dr. Liou Sun, that MAPK pathways are less activated in dwarf cells under stress. Since MAPK pathways are involved in stress sensing (34;35), it would be expected that if the dwarf cells incur less damage from stress, they will sense less damage and will not increase their MAPK signaling. In contrast, it is also possible that changes in MAPK signaling are epistatic to increased Nrf2/ARE signaling in dwarf cells (36), thus defining any pathway between Nrf2 and MAPK signaling would prove informative to several dwarf cellular phenotypes previously observed in the lab, including those from this thesis.

Figure 6.3 depicts a flow chart that summarizes many of the observations reported in this thesis, in the context of the biology and longevity of Snell dwarf mice. At the top, the Snell dwarf mutation at the Pit1 locus causes the abnormal development of the anterior pituitary which leads to the documented hormonal profile of the Snell dwarf mouse. During development, the exposure of cells to this abnormal hormonal profile (e.g. low growth hormone, thyroid hormone, IGF-1 etc.) causes changes (most likely downstream of one of these pathways) that by three months of age, when we remove and culture fibroblasts from the skin of the dwarf mice, are epigenetically stable through several passages in culture. The changes induced by the dwarf hormonal environment, through an unknown pathway, lead to an increase in Nrf2 stabilization and steady-state levels, which lead to an increase in activation of the antioxidant response element. The increased ARE expression leads in turn to increases in several antioxidants and

cellular protection from stress (see Chapter V). The increased resistance to rotenone through increased PMRS activity and resistance to culture in low glucose through increased GSH electron donation are byproducts of the ARE induction, but serve as useful surrogates for these phenotypes. Finally, it is thought that the increased antioxidant protection and stress resistance in cells, if the protection is found in several or all cell types, may protect the organism from damage and slow the aging process.

Future Directions

While the data from this thesis provide significant information relating to differences between cultured cells of long-lived animals (mostly mice), they have several shortcomings and leave a significant number of unanswered questions. An obvious limitation with the work is that the evidence for resistance to low glucose culture and rotenone exposure is entirely correlative in that the metabolic phenomena are observed in long-lived models but lack mechanistic proof for their importance to longevity. This limits the conclusions to the cell culture system and techniques used, preventing them from being applied more broadly at this time. Similarly, data regarding increased Nrf2 levels and ARE expression in dwarf cells are circumstantial in nature, and further work is necessary to prove that Nrf2 signaling is important for the stress resistance of cells from dwarf mice and other long-lived models/species. Furthermore, the connection between cellular stress resistance and increased longevity is also correlative rather than causative, and while it seems plausible that increased cellular stress resistance could prevent the damage that leads to disease in old age, little evidence is available at this time to prove that stress resistance is required for longevity.

Because of the lack of mechanistic information on how non-toxic resistance to low glucose culture or rotenone exposure, increased Nrf2 levels, and cellular cytotoxic stress resistance relate to aging, experiments to formally examine the roles of these phenomena in mammalian aging are difficult to interpret at our

current level of knowledge. Thus, design of experiments on the mechanistic relationship between aging and these cell culture phenomena will require clear information on the respective pathways before they can be tested for their relationship to aging. The work above on Nrf2 and its potential role in all three of these pathways provides a plausible framework for such future studies, but needs to be tested more formally and in more cell types to see whether these observations apply in other settings. It is possible to begin testing the role of Nrf2 in longevity by making mice that overexpress Nrf2 (or express less Keap1), but these experiments will have to circumvent the lethality caused by the significant increase in Nrf2 activity caused by the homozygous Keap1 knockout (37). Despite this, I believe studies of conditional Keap1 heterozygous and homozygous knockouts and/or Nrf2 overexpressors, both time and tissue specific, may be able to provide information as to both when and where Nrf2 may be beneficial to aging. Alternatively, relatively non-toxic Nrf2 activators (e.g. sulforaphane (38;39)) could be used in lieu of genetic mutations, but care would need to be taken to measure the tissues affected by different doses and delivery systems. Unfortunately, studies of this type, especially longevity studies in mice, are very time consuming and expensive, and great care must be taken to describe phenotypes of any conditional mutants before deciding which ones to use for a longevity study. I would suggest, after making the various mice, to expose the mice, and cells explanted from the mice, to a range of organ specific and general toxins, in order to learn which (if any) cells and organs exhibit increased resistance to stress, and to then use the stress resistant mice which have relatively normal overall phenotypes (no developmental or other problems detrimental to health) in lifespan studies. While these assays will not be easy, the difficulty in this work should not discourage its accomplishment using carefully designed experiments to test hypotheses about the role of Nrf2 in mediating longevity.

The work from this thesis can also be extended by additional studies of the dwarf and control-derived primary fibroblasts. Increased steady-state levels of Nrf2

and increased ARE expression have been documented in this thesis, but the mechanism behind the increases and their epigenetic stability in culture remain unknown. Similarly, the mechanisms for activating Nrf2 through pathways other than direct oxidative stress remain unresolved. It is thought that the PI3K, MAPK, and PKC pathways can be involved in Nrf2 activation (27;36;40-42), but the signal transduction pathways are largely unknown. The finding that dwarf fibroblasts, after developing in the abnormal hormonal environment of the dwarf mouse, have a stable increase in Nrf2 levels in culture, suggests that one or more of these hormonal signaling pathways (e.g. insulin/IGF-1, growth hormone, thyroid hormone) may be involved in Nrf2 activation. This information provides a clue as to where to look for upstream signaling and Nrf2 activation in dwarf cells, and suggests that experiments examining individual pathways downstream of the different hormones and upstream of Nrf2 may connect the two pathways and provide information as to the activation of Nrf2. Thus, combining the current knowledge of the dwarf model and of Nrf2 activation may lead to further insight as to how Nrf2 is activated in dwarf fibroblasts, as well as how it might be activated in non-dwarf cells.

Another related area of study in dwarf fibroblasts is to examine the pathways downstream of Nrf2 and its activation. Most studies have focused primarily on Nrf2 activation in terms of its dissociation from binding partner Keap1 (27;43-46), but very little work on how ARE genes are specifically activated has been published. The work in Chapter V shows that in dwarf cells, 4 of the 6 genes examined were upregulated in dwarf cells in the control condition, whereas 2 genes were unchanged between dwarf and control cells. It is likely that a larger panel of genes would show that dwarf cells show increases in many ARE genes, and no increases in others. The reason for specific activation of some parts of the ARE in dwarf cells is unknown, but could involve the mechanism of Nrf2 activation and also possibly the Maf binding partners available to Nrf2 in the nucleus. Using the dwarf fibroblast system we have learned that dwarf cells preferentially activate some ARE genes and do not activate others. Thus,

examining the mechanism of activation (e.g. phosphorylation site, other posttranslational modification) and the levels of Maf binding partners may provide information as to how Nrf2 "selects" one gene over another. Information about downstream effectors of Nrf2 is also sparse. While it is accepted that Nrf2 activity depends on translocation to the nucleus, controlling Nrf2 activity in the nucleus may be important in any treatment model to preferentially activate certain ARE genes over others.

Longevity in mice is probably not due to changes in stress resistance of fibroblasts, even though fibroblasts provide a convenient system for *in vitro* study. While there is substantial circumstantial evidence suggesting that stress resistance in cells increases with longevity in intraspecies (1;3;47;48) and interspecies (4;14) comparisons, the connection between stress resistance and longevity has not been tested formally. However, the idea that increased resistance to cellular stress in many cell types could increase lifespan makes practical sense in that cells protected from damage and death are less likely to cause problems later in life. In post-mitotic cells, such as of neuronal cells or pancreatic cells, protection from stress resistance could prevent the cell death that may lead to late-life diseases such as Parkinson's disease, Alzheimer's disease, and diabetes. In replicating cells, protection from damage could prevent the cellular senescence, loss of stem cell populations, and damage accumulation that leads to the formation of many cancers. Thus it is plausible that cellular stress resistance plays a role in the aging process, by offering protection from the types of damages that accumulate over time.

In order to move toward a more direct test of the hypothesis that stress resistance contributes to increased longevity in dwarf mice, we must eventually test other cells, tissues, and organs both *in vitro* and *in vivo*. It is unknown how many cell types, tissues, or organs will exhibit stress resistance phenotypes in dwarf mice or other models of longevity, but the results may provide clues as to what organs may be more important for any stress resistance related longevity.

Results in stress resistance experiments may also provide clues as to what cell types, tissues, and organs respond to the different hormonal environment in the dwarf mice, and thus advance our knowledge of hormones and development, and suggest pathways that might be disrupted or enhanced to delay the aging process.

Nrf2/ARE activation is frequently described as an adaptive response to both acute and chronic stress (27). The data in this thesis describing increased Nrf2 levels and ARE expression in dwarf fibroblasts imply that increased Nrf2 activation may be behind many of the stress resistance phenotypes observed in the dwarf cells. If this is the case, it raises the question of why cells don't constitutively activate the Nrf2/ARE pathway and thus protect themselves from a variety of damages? The answer to this is unknown, but I speculate that reasons for the evolution of this type of system involve conservation of energy (i.e. it takes a lot of energy to turn on 200+ genes when there without knowing which, if any, will be needed) and the potential side effects of constitutive activation of this system. These side effects include the hyperkeratinosis observed when Keap1 knockout mice develop, which eventually causes their premature death, (37). It is also possible that too large of an increase in Nrf2 signaling could cause cells to increase antioxidant levels so much as to prevent normal cellular ROS signaling, thus creating a loss in cellular redox balance (49). It is notable that just a ~33% increase in Nrf2 levels may be sufficient to increase dwarf cellular ARE mRNA by nearly two-fold, and that this increased ARE may be responsible for many of the ~50-300% increases in stress resistance observed in dwarf cells. Previous work suggests this is plausible, as increased Nrf2/ARE activity causes an increase in cellular stress resistance to glutamate and rotenone in neuronal cells (50), resistance to retinoic acid in hepatocytes (51), and resistance to hydrogen peroxide and peroxynitrite in cardiomyocytes (52). It is possible that greater increases than we observe in dwarf cells could be detrimental to cells because of reasons listed above or other, unforeseen, side effects. Finally, Nrf2 activation has been linked to increased progression of some cancers (53), suggesting that

increased Nrf2 may protect healthy cells, but could be dangerous in abnormal, precancerous cells.

As described in Chapter I, the main goal of this line of research, and of aging research in general, is the eventual development of treatments to delay the aging process in humans. While this work is still somewhat removed from direct aging interventions in humans, work in dwarf mice and other mutants has suggested that disruptions of hormonal pathways, especially the insulin like/IGF-1 pathways, may be effective in slowing the aging process. The work in this thesis, along with work on Nrf2 homologues in invertebrates (54;55), suggests that activation of the Nrf2/ARE pathway could be involved in stress resistance and increased longevity in some organisms. While further work is necessary to explore likelihood that hormonal disruption, Nrf2/ARE activation, or any other intervention (e.g. calorie restriction mimetics) can effectively slow aging in humans, this work represents a small step in the direction of delaying human aging.
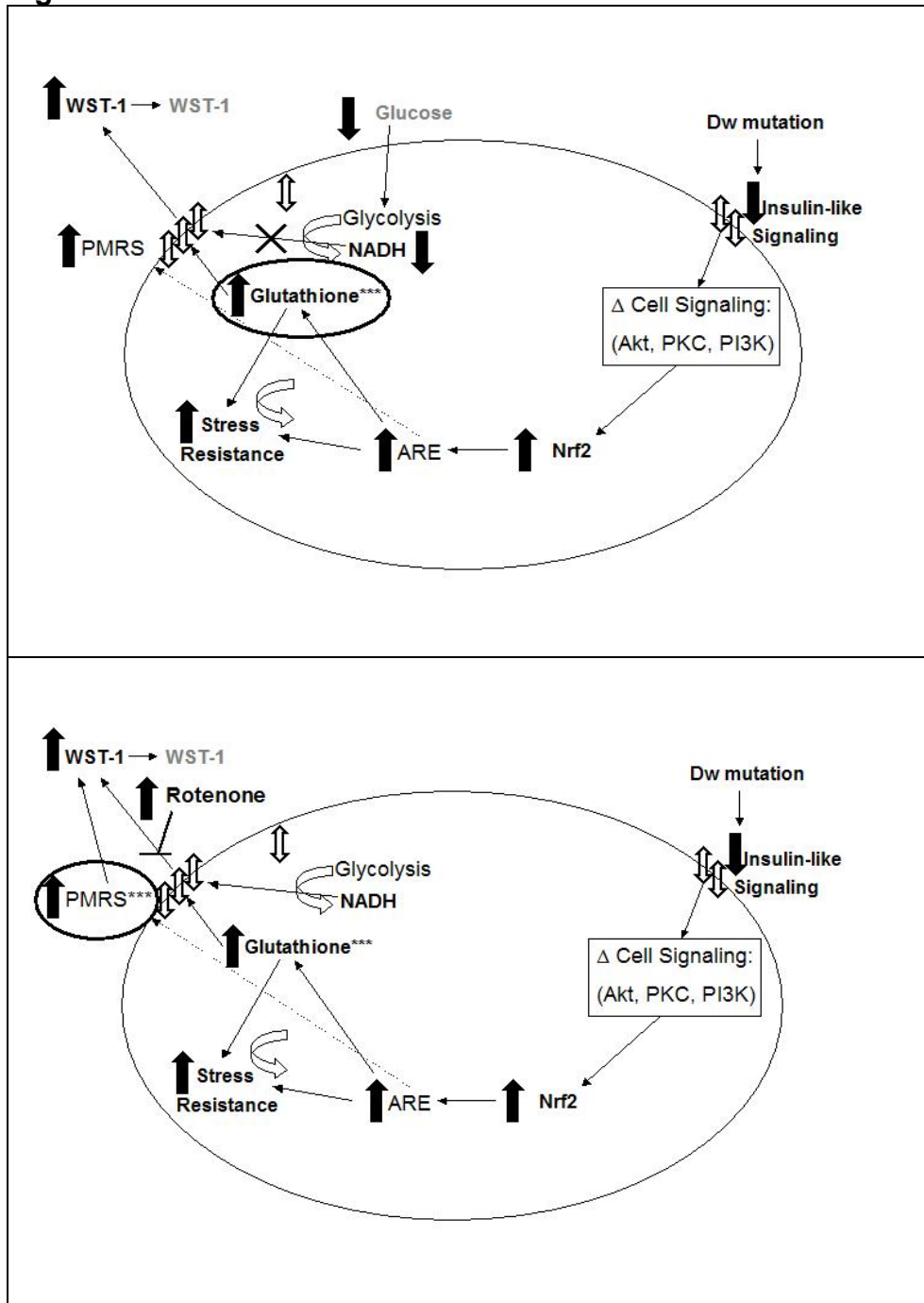
Figure 6.1.
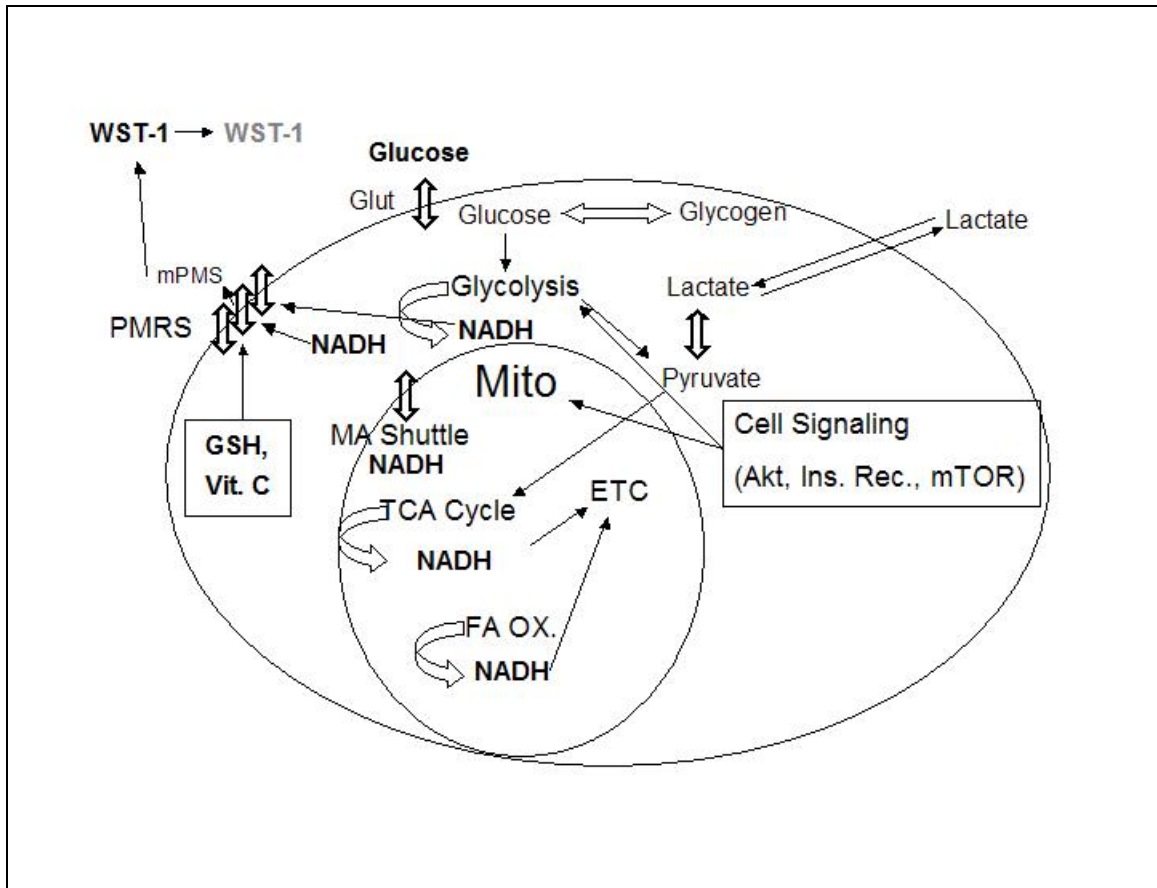
Figure 6.1.   WST-1 Reduction Model.   The model shows how cellular metabolism, and particularly glucose metabolism, might influence reduction of WST-1. Glucose transporters take up extracellular glucose, which is converted to pyruvate in the cytosol to produce ATP and NADH. The pyruvate is then either converted to lactate and released from the cell or taken up by mitochondria and hydrolyzed to produce more NADH and ATP. NADH equivalents can be passed from the mitochondria to the cytosol by the malate aspartate (MA) shuttle, based on their concentration gradient. Together, through an unknown mechanism, NADH molecules (or alternative donors glutathione (GSH) or ascorbate (Vit. C)) reach the plasma membrane, where PMRS enzymes pass electrons from NADH across the membrane and eventually reduce WST-1. All of these metabolic pathways may be modified by signal transduction, including, but not limited to, insulin signaling, Akt, and mTOR signaling (5;56-58).
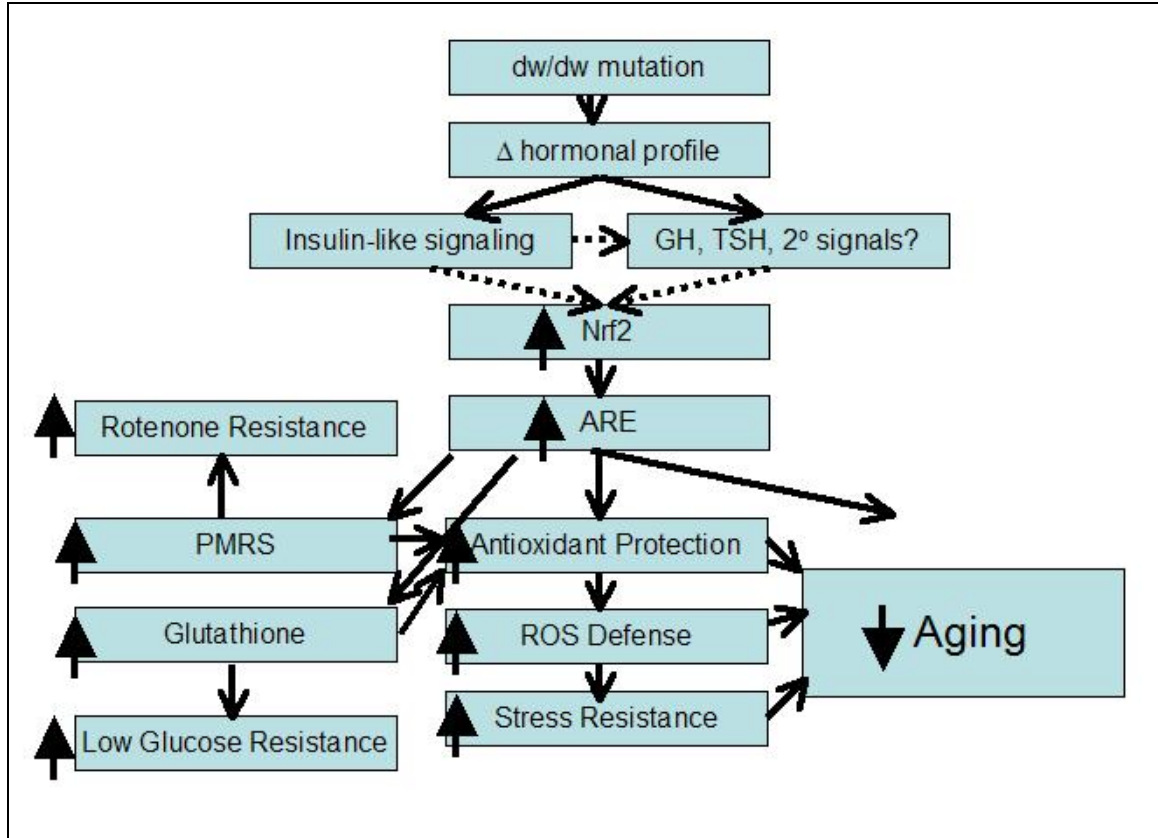
Figure 6.2

Figure 6.2. Postulated Mechanism of dwarf resistance to low glucose culture and rotenone exposure. Figure 6.2 is adapted from Figure 5.7 to depict the specific mechanism of dwarf cells resistance to low glucose medium or to rotenone. Top panel: increased GSH electron donation slows the inhibition of WST-1 reduction caused by decreased NADH levels in low glucose environments. Bottom panel: increased PMRS activity in dwarf cells slows the rotenone induced inhibition of WST-1 reduction acting directly at the plasma membrane.

Figure 6.3.

Figure 6.3. Relationship between Dwarf mutation and aging in a stress resistance model. The model shows a flow chart beginning at the Snell dwarf mouse (dw) mutation, leading to the abnormal hormonal profile of the Snell dwarf mouse, which leads in turn to changes in insulin-like signaling, growth hormone signaling, thyroid signaling, and other secondary changes. These secondary changes, through an unknown mechanism, lead to an epigenetically stable increase in Nrf2 in cultured fibroblast from Snell dwarf mice. The increase in Nrf2 causes an increase in expression of the ARE, which in turn leads to increased cellular glutathione, PMRS activity and antioxidant protection. The increased PMRS activity and glutathione contribute to dwarf cellular resistance to culture in low glucose and rotenone exposure, and together, the differences in antioxidant status and other ARE regulated genes allow for increased defenses against ROS and cytotoxic stress resistance. Cellular stress resistance, if evident in multiple cell types in the dwarf mice, help prevent damage to cells and slow the aging process.

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