**RESEARCH GOALS:** Activating transcription factor (ATF) 4 mediates cellular stress responses. Fibroblast cells from long-lived mouse strains upregulate ATF4 in response to stress more than those from littermate controls[*1*](#_ENREF_1), and cells from long-lived mutant mice are stress resistant[*2*](#_ENREF_2). Likewise, lifespan-increasing treatments elevate expression of ATF4 in mice[*3*](#_ENREF_3). Interestingly, transgenic expression of ATF4 inhibits the mechanistic target of rapamycin (mTOR)[*4*](#_ENREF_4) and increases expression of sirtuin-1 (SIRT1)[*5*](#_ENREF_5), and pharmacological inhibition of mTOR[*6*](#_ENREF_6) or activation of SIRT1[*7*](#_ENREF_7) increase the murine lifespan and stress resistance. However, it remains unclear why ATF4 is upregulated in long-lived or longevity-increased mice and whether increased ATF4 expression mediates the long-lived or stress resistant phenotypes. The research herein proposed will compare how stressors activate stress-response pathways, including pathways that may upregulate ATF4 expression, in long-lived and longevity increased mice *versus* controls. This work will also characterize how chemical or genetic modulation of the activation of those pathways promote or ablate stress resistance. As stress-resistance may be responsible for the long-lived phenotype, identifying mechanisms of increasing stress resistance may also identify methods of increasing mammalian lifespans.

**Aim 1:** Characterize cellular stress pathway activation in long-lived and lifespan-increased mice:

● Measure phosphorylation of eIF2α, HRI, IRE1, PERK, PKR and GCN2, cleavage of ATF6 and mRNA and protein abundance of ATF4, ATF3, CHOP and ASNS in cardiac and skeletal muscle, liver, kidney, pancreas, spleen, lung, brain and fibroblasts from long-lived mouse strains, mice given lifespan-increasing treatments and controls.

**Aim 2:** Chemically induce or ablate cellular stress responses and stress resistance:

● Assay pathway activation as above in conventional mouse fibroblast cells after exposure to increasing doses of stressors such as thapsigarin, tunicamycin, hydrogen peroxide (H2O2), cadmium, ultraviolet (UV) light, methyl methanesulfonate (MMS), and paraquat following treatment with chemical inhibitors or activator of critical pathway components (in table below) or vehicle.

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● Determine stressor LD50s after treatment with vehicle or modulators of critical pathway components.

**BACKGROUND:**

**Stress Response Pathways Upstream of ATF4:** ATF4 translation can be promoted by accumulation of unfolded protein in the endoplasmic reticulum (ER) due to increased mRNA translation, hypoxia and other stresses[*8*](#_ENREF_8). Depletion of protein folding chaperons such as binding immunoglobulin protein (BiP/GRP78) from the ER promotes phosphorylation of protein-kinase R (PKR)-like ER kinase (PERK)[*8*](#_ENREF_8). Activated PERK can phosphorylate eukaryotic elongation factor 2 α (eIF2α), which globally downregulates protein synthesis but increases translation of ATF4 mRNA[*9*](#_ENREF_9). BiP depletion also induces autophosphorylation of inositol-requiring enzyme 1 (IRE1) [*10*](#_ENREF_10). Activated IRE1 catalyzes splicing and translation of X-box binding protein 1 (Xbp-1), which promotes expression of downstream genes[*10*](#_ENREF_10). Activated IRE1 also degrades target mRNAs via regulated IRE1-dependent decay (RIDD) [*10*](#_ENREF_10). Depletion of BiP also promotes relocation of ATF6 from the ER to the Golgi, where ATF6 is cleaved to activate a transcription-factor domain that promotes expression of downstream genes[*8*](#_ENREF_8). Several stresses, including oxidative stress, can induce phosphorylation of PKR and heme-regulated inhibitor (HRI), which can phosphorylate eIF2α. If ATF4 expression is increased due to unfolded protein accumulation, then ATF4 signaling may be accompanied by activation of IRE1, PERK, ATF6, HRI and PKR. Likewise, if ATF4 expression is increased due to activation of PKR and HRI but not due to unfolded protein accumulation, then ATF4 expression may not be accompanied by PERK, IRE1 of ATF6 activation. ATF4 can also be activated by general control nonderepressible 2 (GCN2) kinase due to a variety of stresses[*11*](#_ENREF_11). Phosphorylation of GCN2 is promoted by binding of uncharged tRNAs, which can accumulate due to nutrient deprivation, proteasome inhibition and other processes. GCN2 activation is also promoted by UV irradiation and oxidative stress through poorly understood processes[*11*](#_ENREF_11). Activated GCN2 can phosphorylate eIF2α and promote ATF4 translation[*11*](#_ENREF_11). If ATF4 translation is induced through GCN2 activation, IRE1, PERK and ATF6 may remain inactive.

 **Signaling Downstream of ATF4:** ATF4 modulates downstream gene transcription via several mechanisms[*12*](#_ENREF_12),[*13*](#_ENREF_13),[*14*](#_ENREF_14). ATF4 binds to CCAAT-enhancer-binding protein (C/EBP)-ATF response elements (CAREs) to promote transcription of genes such as ATF3[*15*](#_ENREF_15) and C/EBP homologous protein (CHOP)[*16*](#_ENREF_16). ATF4 can also bind to CAREs in a complex with ATF3[*15*](#_ENREF_15), CHOP[*15*](#_ENREF_15)*,* [*16*](#_ENREF_16), C/EBP and other proteins, to positively or negatively modulate transcription of genes such as asparagine synthetase (ASNS)[*12*](#_ENREF_12), C/EBPβ[*17*](#_ENREF_17), VEGF[*18*](#_ENREF_18) and peroxisome proliferator-activated receptor γ[*17*](#_ENREF_17). ATF4 and many ATF4 target genes regulate expression of upstream proteins[*13*](#_ENREF_13),[*12*](#_ENREF_12).

**EXPECTED RESULTS:**

**Aim 1:** We believe these data will show several eIF2α kinases (e.g., GCN2, HRI, PKR, and PERK) are activated in the tissues or cells long-lived mutant or longevity-increased mice compared to controls. We suspect that other stress-response pathways (e.g. IRE1, ATF6) may be activated in some long-lived mouse strains or by some longevity-increasing treatments. If true, these data will suggest that activation of ATF4 itself rather than activation of parallel pathways may underlie the long-lived and stress resistant phenotypes.

Calorie restriction (CR) and methionine-restriction (Meth-R) can increase the murine lifespan and increase ATF4 expression. We believe CR and Meth-R restriction will induce phosphorylation of GCN2 and eIF2α, consistent with GCN2 detecting increased abundance of uncharged tRNAs, which can be induced by amino acid deprivation or imbalance[*19*](#_ENREF_19). This prediction is supported by reports that eIF2α is rapidly phosphorylated in the brain and liver of mice after consumption of amino-acid imbalanced meal in a GCN2 dependent manner[*20*](#_ENREF_20)*,* [*21*](#_ENREF_21).

Acarbose is an α-glucosidase inhibitor, which reduces nutrient availability from dietary starches and can increase the murine lifespan and increase ATF4 expression, albeit via an unknown mechanism. Acarbose can inhibit lysosomal α-glucosidase and induce lysosomal glycogen storage[*22*](#_ENREF_22). This can perturb intracellular Ca2+ regulation, which may activate PERK directly or indirectly by increasing oxidative stress[*23*](#_ENREF_23). As such, we expect Acarbose treatment will activate PERK and eIF2α, and to possibly activate other oxidative-stress responsive proteins (e.g. PKR and HRI).

Rapamycin is an mTOR inhibitor and chronic rapamycin treatment increases the murine lifespan[*3*](#_ENREF_3) and ATF4 expression in mouse livers[*3*](#_ENREF_3). However, rapamycin either reduces or has no effect on stress-induced ATF4 expression *in vitro*[*24-26*](#_ENREF_24), and short-term rapamycin treatment reduces ATF4 expression in mouse livers[*27*](#_ENREF_27). The reason or reasons for these paradoxical findings is unclear. mTOR inhibition may have cell type specific effects, chronic *versus* short-term rapamycin treatment may differentially regulate ATF4, and ATF4 upregulation may be mediated by processes *in vivo* that are not recapitulated *in vitro*. We expect that long-term rapamycin treatment will induce activation of at-least one of the eIF2α kinases (PERK, PRK, GCN2, HRI) in at-least some mouse tissues.

Decreased circulating insulin-like growth factor 1 (IGF-1) andincreased stress-induced and basal ATF4 expression are characteristic of several long-lived mouse lines, including Snell dwarf mice and pregnancy-associated plasma protein A (PAPP-A) knockout (KO) mice[*2*](#_ENREF_2),[*3*](#_ENREF_3)*,* [*28*](#_ENREF_28).

Circulating IGF-1 is also reduced by CR, Acarbose, and rapamycin[*29*](#_ENREF_29),[*30*](#_ENREF_30). KO of the IGF-1 receptor (IGF1R) and CR both reduce the abundance of BiP, which may promote PERK, IRE1 and ATF6 activation[*31*](#_ENREF_31). However, CHOP or ATF4 expression in IGF1R KO and dwarf mouse fibroblasts cells is either not elevated or only slightly elevated *in vitro*[*1*](#_ENREF_1)*,* [*31*](#_ENREF_31). In contrast, ATF4 and CHOP expression are upregulated in PAPP-A KO and dwarf mouse livers[*1*](#_ENREF_1). As such, we expect ATF4 expression is induced via PERK activation *in vivo*, but suspected that PERK may not be activated and ATF4 expression may not be elevated in long-lived mouse cell lines *in vitro*.

**Aim 2:** We expect stressors will induce ATF4 expression in fibroblasts by activating eIF2α kinases (PERK, PKR, HRI, GCN2). Certain stressors may also activate parallel stress response pathways (ATF6, IRE1). Thapsigarin, cadmium and tunicamycin all disrupt protein folding, either by disrupting ER Ca2+ concentration or N-linked glycosylation. This may activate PERK, IRE1 and ATF6. Paraquat increases mitochondrial production of reactive oxygen species by interfering with electron transport; an effect that can be mimicked by H2O2. This may activate oxidative stress responsive eIF2α kinases (HRI, PKR, PERK). UV light activates the unfolded protein response, induces oxidative stress and activates GCN2[*32*](#_ENREF_32). Finally, MMS is an alkylating mutagen that may activate of GCN2[*33*](#_ENREF_33).

We expect stress induced eIF2α kinase activation can be modulated pharmacologically. Unfolded protein accumulation promotes ATF4 expression via activation of PERK, which can be promoted[*34*](#_ENREF_34) or inhibited[*35*](#_ENREF_35),[*36*](#_ENREF_36). Unfolded protein accumulation also increases ATF4 expression via activation of PKR, which can likewise be promoted[*37*](#_ENREF_37) or inhibited[*38*](#_ENREF_38)*,* [*39*](#_ENREF_39). Oxidative stress can activate HRI (as well as PKR and PERK) and thereby induce ATF4 expression, and HRI activation can be promoted[*40*](#_ENREF_40). Finally, GCN2 activation can be inhibited[*32*](#_ENREF_32), which may reduce ATF4 expression following MMS or UV treatment. We believe augmenting or ablating stressor-induced PERK, HRI and GCN2 activation may respectively increase and decrease stress resistance. However, as PKR signaling can promote cell death, we expect augmenting or ablating stressor induced PKR activation may respectively decrease and increase stress resistance. In contrast, we expect pharmacological modulation of parallel pathways (e.g. IRE1[*41*](#_ENREF_41),) may not affect stress resistance. Finally, we believe pharmacologically promoting phosphorylation of eIF2α[*42*](#_ENREF_42)*,* [*43*](#_ENREF_43) or expression of ATF4[*44*](#_ENREF_44) will increase stress resistance. These predictions are supported by observations that an eIF2α-phosphatase inhibitor increases resistance to tunicamycin*[42](#_ENREF_42" \o "Boyce, 2005 #4852)*, kainic acid[*45*](#_ENREF_45), cadmium[*46*](#_ENREF_46) and rotenone[*47*](#_ENREF_47). Likewise, treatment with fluvoxamine, which induced ATF4 expression, increases tunicamycin resistance[*44*](#_ENREF_44).

**KEY APPROACHES:**

**Aim 1:** The work in Aim 1 involves measuring protein abundance, phosphorylation or cleavage and mRNA abundance in tissue samples preserved from previous experiments or readily available cell lines using established techniques. We will use quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to measure mRNA abundance of ATF4, ATF3, CHOP and ASNS. As some of these proteins (e.g. ATF4) are regulated translationally, the abundance of these proteins will also be measure by western blot. We will also assay the activation of PERK, HRI, PKR, and GCN2 by comparing the abundance of these proteins and their phosphorylated isoforms by western blot. Likewise, we will measure the relative abundance of full-length and cleaved ATF6 by western blot to assay ATF6 activation.

**Aim 2:**  The work described in Aim 2 involves measuring the LD50 of numerous stressors, the effect of these stressors on stress response pathway activation, and the effects of chemical modulators of stress-response proteins on stressor LD50 and stress response pathway activation. This work uses mostly established techniques, or requires minor modifications of such techniques. Protocols for measuring the LD50 of UV light, H2O2, cadmium, paraquat, and MMS in mouse fibroblasts have been described in our lab, and measuring the LD50 of thapsigarin and tunicamycin in cell culture have been described elsewhere. Likewise, the activity of the chemical modulators has been investigatedin a variety of cell lines, but not always in mouse fibroblasts.

**IMPLICATIONS:**

**Aim 1:** Theresults of Aim 1 may support the hypothesis that the long-lived and stress-resistant phenotypes of long-lived and longevity-increased mice stems from increased ATF4 expression. These results may alternately suggest that parallel pathway activation (e.g. IRE1 and ATF6) may underlie these phenotypes. Regardless, identifying the pathway or pathways that are activated in cells that possess these phenotypes may elucidate novel methods of inducing or ablating these phenotypes. Inducing a stress-resistant and long-lived phenotype is of considerable gerontological concern, while ablating stress resistance may sensitize malignancies to chemotherapeutics.

**Aim 2:** The results of Aim 2 may identify novel mechanism of inducing the stress-resistant phenotype that occurs in long-lived and longevity-increased mice. These phenotypes may be due to increased ATF4 expression following eIF2α, HRI, PKR, PEKR, or GCN2 activation. Alternately, they may stem from activation of IRE1 or ATF6 by stressors that only incidentally increase ATF4 expression. By modulating the activity of these proteins in stressed and unstressed conditions we may determine whether their activation is necessary or sufficient to mediate stress resistance. Likewise, these data will reveal if these modulators affect off-target pathways.

**PROFESSIONAL DEVELOPMENT:**

 The skills and techniques involved in this project are generalizable rather than narrowly tailored, and will be useful in the applicant’s future career. This project will provide the applicant experience in performing qRT-PCR and western blotting using established protocols, and the opportunity to apply those skills to develop novel qRT-PCR protocols and validate the activity of untested but commercially available antibodies. Likewise, this project will provide the applicant the opportunity to learn new cell-culture techniques including the derivation of mouse fibroblast cell lines and determination of cellular viability following stress induction or drug treatment. These techniques can be feasibly conducted using the equipment, reagents and expertise of the mentor, his staff or his collaborators. Finally, the results of this project may provide a basis for continued work in our lab or elsewhere, and may make valuable contributions to the body of scientific literature.

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