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Dr. Pletcher,

 Included herein is a progress report for my T32 project ‘Investigating Pharmacological Induction and Ablation of a Stress-Resistant Phenotype Characteristic of Long-Lived Mice.’ This project sought to expand upon initial work by Dr. Weiquan Li, which showed that fibroblast cells from long-lived mouse strains upregulate ATF4 in response to stress more than those from littermate controls[*1*](#_ENREF_1), and that lifespan-increasing treatments elevate expression of ATF4 in mice[*2*](#_ENREF_2).

The first aim of this project was to ‘characterize cellular stress pathway activation in long-lived and lifespan-increased mice.’ We sought to achieve this aim by investigating upstream signaling events that promote ATF4 mRNA translation in Snell dwarf and control mice. ATF4 mRNA translation is paradoxically promoted during periods of global mRNA translational repression. The most well characterized mechanism of global translational repression occurs via phosphorylation of eukaryotic translation initiation factor 2 α (eIF2α) at serine 51. Interestingly, while we were able to reproduce previous findings that the abundance ATF4 protein was elevated in the livers of Snell dwarf mice, this increase was not accompanied by a significant increase in phosphorylation of eIF2α at Serine 51 (Figure 1). This finding proved robust, with hepatic eIF2α phosphorylation in dwarf mice being either lower or not significantly different from control mice in several analyses that varied nutritional status and which tested multiple anti-phospho-S51-eIF2α antibodies (data not shown).



***Figure 1:******Elevation of ATF4 in the livers of Snell dwarf mice is not due to increased eIF2α phosphorylation. (A)*** *Representative lanes of an immunoblot detecting ATF4, β Actin, eIF2α or eIF2α phosphorylated at serine 51 in the livers of six each male and female Snell dwarf and control mice.* ***(B)*** *Quantification of hepatic ATF4 expression.* ***(C)*** *Quantification of hepatic eIF2α phosphorylation. Error bars show standard deviation, and significant differences (\*) were detected via a two-way independent samples t-test.*

This unexpected finding contradicted the premise of Aim 1; that increased ATF4 protein abundance would be due to changes in eIF2α phosphorylation, which might likewise be due to differences in upstream eIF2α kinase activity. We investigated several eIF2α independent mechanisms by which ATF4 translation or stability might be increased, but these analyses revealed either no differences between dwarf and control mice or that commercially available reagents were of insufficient quality to conduct the desired analysis (data not shown). In light of these findings we determined that the scope of the project had expanded beyond the narrow investigation of well-characterized pathways we initially proposed, and into an open-ended investigation of improbable or potentially untestable *ad hoc* hypotheses. We thus decided to re-focus our efforts on another narrowly defined and achievable project.

The Miller laboratory and other labs have reported that dietary restriction of the essential amino acid methionine can extend the murine lifespan[*3*](#_ENREF_3)*,* [*4*](#_ENREF_4). Interestingly, such treatments can increase the lifespan of control mice up to that of certain long-lived mice, but the lifespan of such long-lived mice is not itself altered by methionine restriction[*5*](#_ENREF_5). This may be due to differences in methionine metabolism between long-lived and control mice[*6*](#_ENREF_6)*,* [*7*](#_ENREF_7), or due to more general differences in how cells from such mice respond to amino acid restriction, previously reported by Wang and Miller[*8*](#_ENREF_8). These findings suggest that the differences in how dwarf and control mice respond to amino acid restriction or deprivation may also underlie the longer lifespans generally observed in such dwarf mice. In addition, unpublished work (Li, Miller) has shown that cells from long-lived primates have differing responses to amino acid withdrawal compared to cells from short-lived primates. It is not known whether these responses represent sensitivity to methionine, or to other amino acids, and it is not known how these differences are regulated intracellularly.

I have therefore begun to investigate how dwarf and control fibroblasts respond to total and selective amino acid withdrawal using established techniques adapted from work in our laboratory by Dr. Min Wang[*8*](#_ENREF_8) and guided in part by similar characterization in other cell lines[*9*](#_ENREF_9)*,* [*10*](#_ENREF_10). Following selective or total amino acid withdrawal *in vitro* from dwarf and control fibroblasts we will investigate amino-acid sensing and responsive pathways, including the mTOR pathway and the eIF2α-ATF4 pathway[*11*](#_ENREF_11). These pathways are of particular interest to our lab given their implication in mouse aging. Additionally, I have experience with several of the assays necessary to characterize activation of the eIF2α-ATF4 pathway from my previous project. Likewise, Dr. Gonzalo Garcia in the Miller lab has the requisite experience and expertise to give me guidance on analyzing portions of the mTOR pathway in these experiments[*12*](#_ENREF_12).



***Figure 2: Dwarf (DWR) and control (CTRL) fibroblasts respond to amino acid deprivation in vitro.*** *Cells were cultured in either modified Eagle’s media (MEM), or were deprived of amino acids by incubation in Earl’s balanced salt solution (EBSS) for one hour, or were deprived of all but one essential amino acid, which was added into EBSS to the same concentration found in MEM (+Met, +Leu,+Val).* ***(A)*** *Representative blots of analyzed proteins.* ***(B)*** *Quantification of relative eIF2α phosphorylation.* ***(C)*** *Quantification of relative ribosomal S6 protein phosphorylation.*

 We have conducted some preliminary experiments to demonstrate the feasibility of this project (Figure 2). Following amino acid restriction *in vitro* we would expect that eIF2α phosphorylation should increase, and this result can be reliably detected in both dwarf and control fibroblasts (Figure 2B). In contrast, following amino acid restriction we would expect to see a reduction in ribosomal S6 protein phosphorylation, which is likewise detectable in both dwarf and control fibroblasts (Figure 2C). We are running additional preliminary experiments in order to further optimize and validate these and other assays. Our immediate priority is to recapitulate Dr. Wang’s[*8*](#_ENREF_8) finding that dwarf and control cells respond differently to total amino acid withdrawal.

In the future we plan to test the withdrawal of each of the eight essential amino acids, as conducted in previous investigations[*9*](#_ENREF_9)*,* [*10*](#_ENREF_10). We additionally plan to test the withdrawal all-but one of the essential amino acids (as in Figure 2). These two analyses will allow us to determine whether dwarf and control cells are differentially responsive to selective amino acid withdrawal rather than the total amino acid withdrawal Dr. Min Wang investigated[*8*](#_ENREF_8). These results may be useful for understanding mechanisms that may underlie the increased lifespan that is observed following amino acid restriction in mice *in vivo*. Moreover, we are ideally position to follow up our *in vitro* findings *in vivo* using samples from previous and ongoing amino acid restriction experiments under way in the Miller laboratory.

 This project is technically feasible and draws upon our lab's previous work, current expertise and future plans. In addition to generating novel and scientifically interesting findings, this project may allow me to gain additional experience in cell culture. I appreciate your consideration for continued support of my training and research.

Regards,

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