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CHARACTERIZATION OF THE MITOCHONDIRAL UPR-REGULATED SERPIN SRP-1

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by

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ABSTRACT

CHARACTERIZATION OF THE MITOCHONDIRAL

UPR-REGULATED SERPIN

SRP-1

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The mitochondrial unfolded protein response (UPRmt) is a regulatory stress response that is involved in the protection of mitochondrial function. Cells activate this transcriptional response to promote cell survival and the recovery of the mitochondrial functionality. In the model organism *Caenorhabditis elegans*, the UPRmt is regulated by an essential transcription factor known as ATFS-1. When mitochondrial function is compromised, ATFS-1 regulates the expression of a variety of genes, including *srp-1*, which encodes an uncharacterized serpin. Serpins are part of a larger superfamily with classical roles in blocking protease enzymes, but their function during mitochondrial stress is unknown. We sought to examine the role of this UPRmt-regulated serpin during

mitochondrial stress. We find that *srp-1* gene knockdown by RNA interference enhances UPRmt activation during mitochondrial stress. Interestingly, *srp-1* displays a refined expression in a subset of four small interfacial epithelial cells. Furthermore, *srp-1* protein appears to localize to the endoplasmic reticulum suggesting inter-organellar stress crosstalk.

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CHAPTER 1

INTRODUCTION

1.1 Quality Control within Mitochondrial Dysfunction

Mitochondria are double membrane organelles believed to have arisen from the engulfment of an alpha proteobacterium by a pre-eukaryotic cell about 1 billion years ago. These “powerhouses” of the cell are noted for generating most of the cell’s energy through the actions of oxidative phosphorylation. However, they have additional important roles including energy homeostasis, metabolism, and apoptosis. Furthermore, these organelles serve to synthesize amino acids and regulate innate immunity.

Mutations in mitochondrial genes results in buildup of damaging reactive oxygen species that impair the both mitochondrial proteome and genome and are the main cause for mitochondrial disorders. In fact, increasing mitochondrial damage is associated with normal organismal aging as well as the development of various diseases.

Due to the importance of mitochondria and the various challenges it faces, there are mechanisms in place to help protect the organelle. One such mechanism is the mitochondrial unfolded protein response (UPRmt). The UPRmt is a stress responses pathway that is activated during mitochondrial dysfunction to ensure repair and recovery of the intricate mitochondrial network. The UPRmt is mediated by the bZIP transcription factor ATFS-1 (Activated Transcription Factor under Stress) that is involved in the

transcriptional regulation of hundreds of genes involved predominantly in mitochondrial repair. As well, the UPRmt regulates a host of innate immunity genes to help protect the host during infection with those pathogens that target mitochondrial function. ATFS-1 has an intriguing protein structure containing both a nuclear localization sequence (NLS) and a mitochondrial targeting signal sequence (MTS). Healthy mitochondria import ATFS-1 and degrade it by the Lon protease. When mitochondria are stressed, ATFS-1 cannot be imported into mitochondria since the mitochondrial inner membrane potential and ATP levels are reduced. Instead, ATFS-1 translocate to the nucleus to transcriptionally regulate approximately 400 genes mostly with mitochondrial protective properties. Once mitochondrial function is restored, import of ATFS-1 into mitochondria resumes where it is degraded by a protease in the mitochondrial matrix as a means of inactivation since it is no longer needed.

1.2 Mitohormesis within *C. elegans*

The mitohormesis model proposes that the reactive oxygen species (ROS) produced by the mitochondria increases the longevity of an organism, and that this correlation is important for the lifespan. In other words, mitochondrial stress triggers a hormetic response providing short term metabolic benefits as well as long term benefits in increased stress tolerance.

As the UPRmt acts as a stress response pathway to repair damaged mitochondria and is activated under conditions that drive mitohormesis, it is thought to play a role in this mechanism of increased longevity. However, this relationship is not without controversy. While some groups have found that loss of UPRmt regulators suppresses the extended lifespan benefit of mitohormesis, others have found no difference. The activation of the

UPRmt undoubtedly correlates with mitohormesis conditions but it also correlates with conditions that do not provide a lifespan benefit or in fact reduces lifespan. Also, a gain-of-function allele in *atfs-1* that prevents import mitochondria leading to constitutive activation of the UPRmt reduces lifespan instead of enhancing it.

Nonetheless, we sought to further dissect the possible involvement of the UPRmt in mitohormesis longevity. We examined the complete list of ATFS-1 regulated genes for potential involvement in regulating the UPRmt and longevity. Briefly, we knocked down expression of a subset of genes by double stranded RNA interference (RNAi) and examined their effect on UPRmt activation. Doing so, we discovered that the uncharacterized serpin gene *srp-1* showed an obvious effect on UPRmt activity. SRP-1 is the *C. elegans* homolog of human SERPINB5, a class of protease inhibitors.

Because ATFS-1 is a regulator of the *srp-1* gene, we suspect that the knockdown of *srp-1* will affect the reaction of the worm to mitochondrial stress. Therefore, this may impact the increased longevity of animals that experience mitohormesis.

CHAPTER 2

METHODOLOGY

We examined the effect of *srp-1* knockdown on UPRmt activity using the SJ4100 *C. elegans* transgenic strain that harbors a transcriptional green fluorescent protein reporter for hsp-6, a mitochondrial chaperone gene that is regulated by ATFS-1 during the ATFS-1. To enhance the effectiveness of the RNAi, we used a mutant in the *rrf-3* gene.

Lifespan analysis was conducted using the *clk-1* mutant background, which contains a reduction of function mutation in a component important for the function of the mitochondrial electron transport chain. Disruption of *clk-1* leads to elevated ROS levels that engages mitohormesis allowing the worm to live significantly longer than wild-type. *rrf-3* and *clk-1;rrf-3* mutants were fed *E. coli* expressing the double stranded RNA for the *srp-1* gene to knock down its expression. As a negative control, worms were fed the *E. coli* bacteria with an empty plasmid (called "vector"). The *E. coli* bacteria where *srp-1* is inserted contains adjacent bacterial inducible T7 promoters. In order to stimulate the transcription of the gene on both strands of the DNA to generate double stranded RNA (dsRNA), a T7 promoter chemical inducer (IPTG) will be added. Because of RNA's normal state as a single stranded molecule, the cell will identify the dsRNA as foreign and cleave it into 22 small nucleotide fragments. These small fragments will finally bind to a corresponding gene on the endogenous RNA transcripts leading to its degradation.

The worms were fed the RNAi *E.coli* which allowed for the knockdown of the *srp-1* gene. Worms were allowed to grow to the adult stage at which time they were transferred

onto fresh RNAi plates each day. This was done to ensure that they did not starve, and for counting purposes of the worms to avoid the inclusion of eventual progeny.

We also monitored the effect of *srp-1* knockdown on the survival of the host to pathogen infection. This was performed since the UPRmt was shown to protect the host during infection and that pre-activation of the UPRmt (e.g. using the *clk-1* mutant) prior to infection extends their survival. Survival assays were also performed using a pathogenic bacterium, infectious *Pseudomonas aeruginosa*.

We next examined the expression of *srp-1* in the whole *C. elegans* animals by creating a transcriptional GFP reporter. This construct consisted of approximately 2.5 kb of upstream promoter sequence fused to GFP. This construct was microinjected into wild-type animals along with a transformation marker *myo-2*::mCherry. Stable transgenic lines were obtained and viewed using fluorescent microscopy.

To probe the localization of *srp-1* in the cell, we cloned the *srp-1* cDNA into a mammalian expression plasmid to view the localization in mammalian cells grown in culture. This was performed to allow for better resolution of cellular structures compared to that that is observed in *C. elegans*. The *srp-1* cDNA was cloned into the pEGFP-N1 mammalian expression plasmid and then transfected into the cell line 293-T using Lipofectamine.

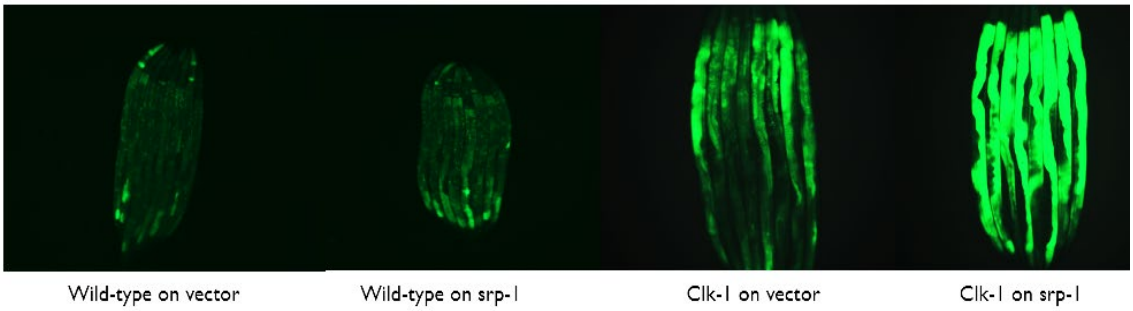
CHAPTER 3

RESULTS

3.1 Effect of *srp-1* Knockdown on the Activity of the UPRmt

We knocked down the expression of *srp-1* both in healthy animals and the *clk-1* mutant which bears mild mitochondrial stress. We did not observe any difference in expression of the *hsp-6*::GFP in srp-1(RNAi) animals. As expected, the UPRmt was activated in *clk-1* mutants but interestingly, *srp-1* RNAi further enhanced the activity of the pathway.

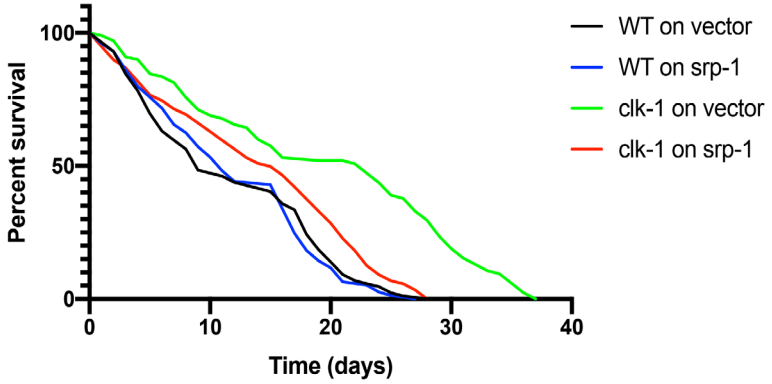
Fig 3.1: *srp-1* knockdown enhances UPRmt



3.2 Lifespan Assay of *C. elegans*

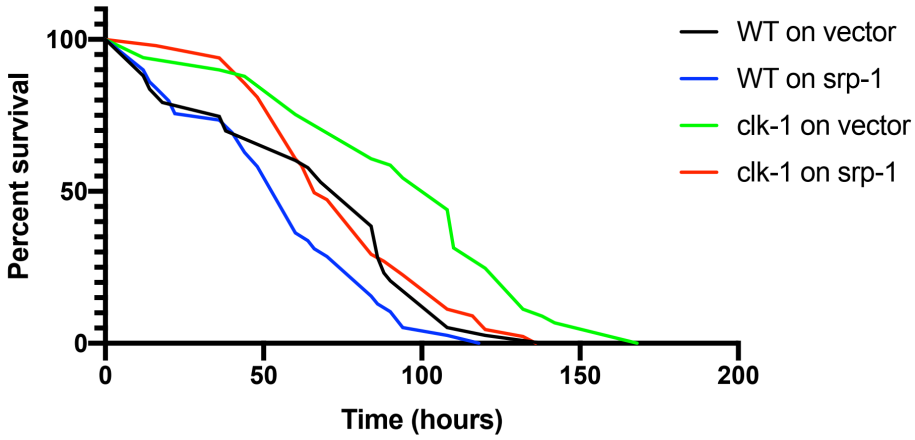
We next monitored the lifespan of clk-1 mutants in the presence or absence of *srp-1*. As expected, *clk-1* mutants lived longer than wild-type animals because of mitohormesis. RNAi against *srp-1* significantly reduced the lifespan of *clk-1* mutants. Importantly, *srp-1* RNAi had no effect on the lifespan of wild-type animals. This result suggests that *srp-1* specifically mediates the enhanced longevity offered through mitohormesis and is not required for general viability of the animal.

Figure 3.2: Knockdown of *srp-1* reduced the lifespan of long-lived mitohormetic *C. elegans*



3.2 Survival assays of *C. elegans* during infection with pathogen

We next measured the survival period of animals challenged with pathogen infection. As shown previously, *clk-1* mutants lived longer when infected with the pathogen *P. aeruginosa*, likely due to the activation of the UPRmt. Similar to the effect on longevity, *srp-1* RNAi significantly reduced the survival of *clk-1* mutant animals during infection. Strikingly, *srp-1* RNAi significantly reduced the survival of healthy animals when exposed to *P. aeruginosa*. Thus, *srp-1* is required to mediate the survival benefit during infection with *P. aeruginosa*.



3.3: Knockdown of *srp-1* reduced survival of *C. elegans* during infection

3.3 Expression of the *srp-1* gene within *C. elegans*

In order to find out where the *srp-1* gene is expressed in *C. elegans*, we prepared a *srp-1*::GFP construct and injected into wild-type worms. Although the expression was extremely low, we did detect *srp-1* in the intestine of the worm and two epidermal cells in the head region which we believe to be the arcade cells. (Figure 3.2).

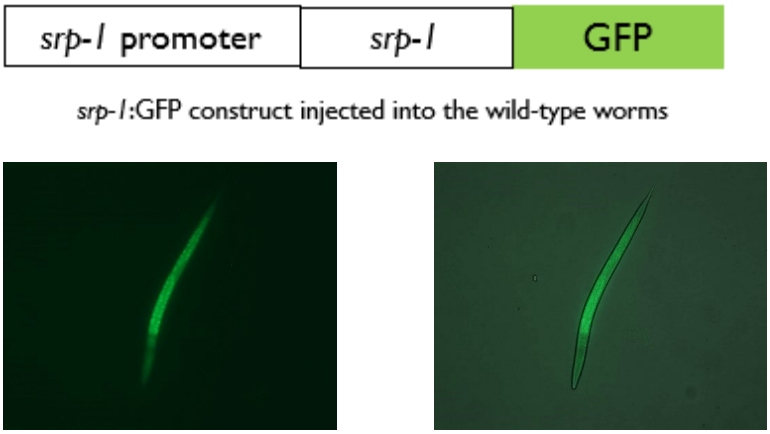


Figure 3.4: GFP fluorescence as depicted in the intestinal cells of the wild-type worms

3.4 Localization of the *srp-1* gene within HEK cells

The localization of the *srp-1* was examined by expressing a SRP-1::GFP translational fusion protein in mammalian cells. Interestingly, the SRP-1::GFP fusion protein displayed a diffuse localization pattern with an intermittent perforation pattern. The localization pattern was quite reminiscent of proteins that localize to the endoplasmic reticulum sheets which contain rough ER.

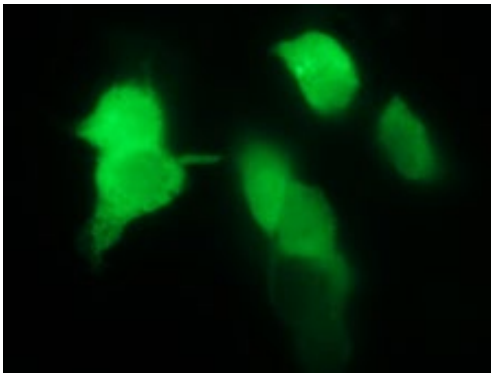


Figure 3.5: GFP fluorescence of the *srp-1* shows localization in the endoplasmic reticulum of the human embryonic kidney cells

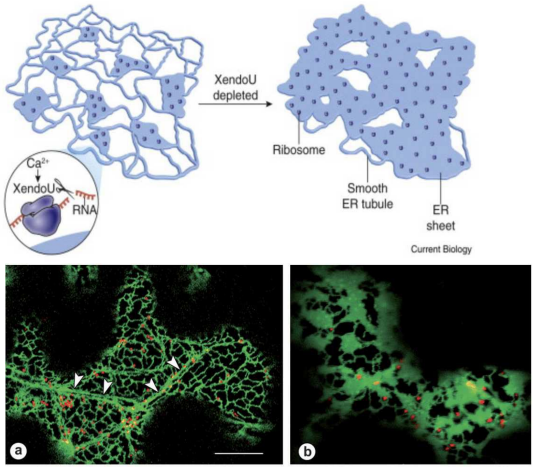


Figure 3.6: Diagram of ER sheets

CHAPTER 4

DISCUSSION

4.1 Significance of Study

Mitochondrial function declines as an organism age. But counterintuitively, mild mitochondrial stress early in life leads to extended lifespan via mitohormesis through the activation of protective pathways such as the UPRmt. In this study, we discovered that the UPRmt-regulated gene *srp-1* mediates the mitohormesis increase in longevity. Knockdown of *srp-1* by RNAi further enhanced the UPRmt and reduced the lifespan of *clk-1* mutants that normally live longer than wild-type animals. Presumably, the increase in UPRmt activity is due to added mitochondrial stress that goes beyond the mild threshold of mitohormesis. As a result of the stronger mitochondrial stress the animal showed reduced lifespan. What is critical is the fact that *srp-1* RNAi did not reduce the lifespan of otherwise healthy normal animals. This means that *srp-1* mediates specifically the effect observed due to mitohormesis and is not an essential gene for normal survival. Also, this is consistent with *srp-1* being transcriptionally induced during the UPRmt by ATFS-1. In addition, we observed a similar trend during infection of *clk-1* mutants with the pathogen *P. aeruginosa*. However, this time *srp-1* RNAi reduced the survival of otherwise healthy animals. This is because *P. aeruginosa* infection caused stress to mitochondria thus activating expression of srp-1.

It is also very intriguing that *srp-1* localizes to the ER yet has such an obvious impact on mitochondrial stress signaling and mitohormesis. If true, then this would

represent a novel player in mitochondrial-ER crosstalk. Also, this would imply that ATFS-1 mediates the transcriptional regulation of a gene (*srp-1*) that plays a role in ER maintenance or function. This would be the first example of this type of regulation by ATFS-1. Considering that mitochondria and ER are physically interacting organelles that share many functional roles, the discovery of *srp-1* adds a further layer depth of this inter-organellar communication.

4.2 Follow-up Experiments

Much is still needed to understand the role of *srp-1* in mediating mitohormesis. First, what is the function of the *srp-1* protein? As a predicted serpin, it should bind and inhibit protease(s). We would need to identify which substrates are directly bound by *srp-1* using protein purification and mass spectrometry. Second, how does *srp-1* impact ER maintenance and function? We can use a variety of ER markers and measure how they change in the presence of mitochondrial stress in the presence or absence of *srp-1*. Lastly, what role does ATFS-1 play in ER maintenance? For instance, are there other ATFS-1-dependent genes that mediate the UPRmt and mitohormesis longevity?

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BIOGRAPHICAL INFORMATION

Sedra Albosstani will be graduating in the Spring of 2019 with an Honors Bachelor of Science in Biology. She seeks to pursue a career in medicine and is considering continuing research in the field of science until she begins her medical journey. She has served in many nonprofit and student organizations, including presiding over the Muslim Student Association for two terms, serving on the board of the Student National Medical Association, and the board of the Medical Dental and Preparatory Association. Awarded with a recognition of participation, Sedra also took part in organizing and participating in UTA's first medical hackathon.

Sedra has conducted research in a biology lab for Dr. Johnathan Campbell, prior to joining Dr. Mark Pellegrino's lab. She seeks to use her expertise in the lab for a possible future as an MD-PHD student.