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**Role of metabolic shifts in protection from mutation damage:
Characterizing mitochondrial membrane potential in *C. elegans*
gas-1 mutants**

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Abstract

Many terminal human diseases are caused by mutations affecting mitochondrial functioning. Mitochondria are essential organelles responsible for producing cellular energy, **adenosine triphosphate (ATP)** via **oxidative phosphorylation (OXPHOS)** at mitochondrial **electron transport chains (ETC)**. Proper ETC functioning relies on maintenance of the electrochemical gradient essential for energy production, known as **mitochondrial membrane potential ($\Delta\psi$ M)**. The inner mitochondrial membrane is the site of the ETC and is most closely in contact with the enzymatic processes occurring within the mitochondrial matrix. Mutations affecting protein components of the ETC are especially troublesome for organelle health. ETC mutants commonly express altered $\Delta\psi$ M, as well as increased production of damaging **reactive oxygen species (ROS)**, which are hypothesized to cause genomic damage and lasting mutation. The nematode *C. elegans* is a practical model organism for investigating the phenotypic and genomic consequences of ETC mutations. Despite expressing higher levels of damaging ROS, the ETC protein complex 1 mutant, *gas-1*, expresses heritable mtDNA and nDNA mutation rates identical to those of wild type animals. I am using a mitochondria-targeted dye and fluorescence microscopy to quantify and compare $\Delta\psi$ M levels of the *gas-1* mutant and wild type animals. This work will provide a novel phenotypic characterization of this mutant and indicate whether decreased metabolic activity (e.g., reduced reliance on OXPHOS) is occurring in *gas-1*, and perhaps conferring protection from genomic degradation. Based on *gas-1*'s characteristically low ATP production and high ROS production, I expect that $\Delta\psi$ M will be higher in *gas-1* as compared to wild type levels.

Introduction

Mitochondria are arguably the most important organelle to cellular health and performance because they are the site of bioenergy metabolism for the entire cell. Glucose is digested and sent to the Citric Acid Cycle (TCA) located in the mitochondrial cytoplasm in the form of pyruvate, which is then metabolized to provide electrons in the form of NADH and FADH₂ for use in oxidative phosphorylation (OXPHOS) occurring at the mitochondrial electron transport chain (ETC). The ETC is comprised of a series of proteins, organized into supercomplexes, located along the inner mitochondrial membrane. The movement of electrons through the complexes of the ETC produces more cellular energy in the form of ATP than glycolysis and the tricarboxylic acid cycle (TCA) cycle combined, and is powered by the constant movement of ions across the inner membrane, known as an electrochemical gradient, or the inner mitochondrial membrane potential ($\Delta\psi_M$).

Mutations in both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) can be responsible for coded dysfunction in a mitochondrion. Mutations affecting the ETC will consequently inhibit the production of ATP via OXPHOS and possibly other important regulatory functions of mitochondria. In addition to its central role in ETC and cellular function, maintenance of $\Delta\psi_M$ is crucial for the process of mitochondrial fusion, and excessive fragmentation (fission) of mitochondria results when $\Delta\psi_M$ is disrupted due to overwhelming proton translocator activity (Legros, 2002). Cycles of mitochondrial fission and fusion are crucial to overall cell health; however, only mitochondrial fusion is dependent upon $\Delta\psi_M$ (Legros, 2002). Because mitochondrial function is crucial for energy metabolism, dysfunctions within these organelles can lead to a broad range of diseases in animals, including Parkinson's disease in humans (Celardo, Martins, and Gandhi, 2014).

Complex 1 of the ETC is specifically responsible for the oxidation of NADH to lone electrons and protons and creating the initial proton gradient along the inner mitochondrial membrane, which initiates energy production through OXPHOS. This movement of ions is the main source of reactive oxygen species (ROS) due to electron leakage onto molecular oxygen, which is ever present due to the nature of this process (Hicks, Denver, and Estes, 2013). Although some ROS are critically important for molecular signaling, at high levels they are damaging to proteins, can cause morphological changes in mitochondria, disrupt $\Delta\psi_M$, delay organismal development, and cause oxidative damage to DNA and lasting mutation (Shibuya and Tsujimoto, 2012). The idea that ROS-induced oxidative damage is an important source of somatic (non-gamete) or heritable (germline) mutation in nDNA or mtDNA genomes is controversial (Griffiths et al., 2000).

Altered metabolic processes were first observed in colorectal cancer cells (Warburg et al., 1956). The Warburg Effect is a phenomenon characterized by a shift in metabolism from primary use of OXPHOS to a less oxidatively stressful (i.e., lower ROS producing) but much less energetically efficient mixture of aerobic glycolysis and OXPHOS, which benefits rapidly proliferating cells (1956; Lunt and VanderHeiden, 2011). Ericson et al. (2012) measured the frequency of random de novo single base substitutions in the mtDNA of human colorectal cancer cells and discovered that tumor tissue had fewer C:G to T:A transition mutations (known to be associated with oxidative damage) compared to healthy colorectal tissue cells. Ericson et al. (2012) also showed that this decrease in mutation frequency was coupled with a metabolic shift from OXPHOS to anaerobic glycolysis, which was not witnessed in healthy colorectal cells. These findings suggest that the stability of the mitochondrial genome is increased in cancer compared to healthy cells, as a result of a decrease in ROS related mtDNA damage. Inhibited or suppressed energy production has also been observed in a wide variety of other diseases, including diabetes and neurodegenerative disorders (DeBerardinis, 2012). However, whether and to what extent altered or depressed metabolism can protect organisms from heritable (germline) mutations has not been studied.

C. elegans nematodes are becoming an increasingly important model system for studying mitochondrial dysfunction and its relationship with energy metabolism and mutation. These

microscopic animals are easy to maintain, transparent, non-parasitic, and can be used for studying the effects of mutation on individuals and populations with relative ease. Among several ETC mutant alleles available for *C. elegans*, *gas-1 (fc21)* is particularly well-studied. *gas-1* is a nuclear gene and *gas-1 (fc21)* is a single base pair substitution that creates a hypomorphic allele in a protein component of ETC complex 1 (http://www.wormbase.org/species/c_elegans/gene/WBGene00001520?query=gas-1#0-9e-3). *gas-1* expresses many altered physiological phenotypes such as increased dependence on ETC complex II to maintain metabolic homeostasis, decreased growth rate, decreased fecundity, a characteristic hypersensitivity to volatile anesthetics, increased levels of ROS, and decreased OXPHOS abilities as compared to that of wild type (Kayser et al., 2001). Despite the fact that *gas-1* mutants exhibit elevated levels of damaging ROS, previous studies performed by the D. R. Denver lab (Oregon State Univ.) have concluded that both mtDNA and nDNA mutation rates in *gas-1* are identical to those expressed by wild type animals (Estes pers. comm.). This result may indicate that ROS do not affect heritable mutation rates as previously thought. Based on these and previous findings, we believe that *gas-1* is experiencing a shift in metabolism synonymous with the Warburg Effect, and as a result, is gaining genomic protection from the mutagenic effects of high levels of ROS by reducing its dependence on energy production via the OXPHOS pathway (c.f., Ericson et al., 2012).

Previous phenotyping of *gas-1* has included various life-history and physiological traits (Kayser et al., 2001; Cairo et al., 1992; Estes et al., unpubl.), and studies of gene expression (Falk et al. 2008); however, $\Delta\psi M$ has not yet been characterized. Further investigation of *gas-1* is necessary to understand the effects of mutation on the ETC metabolic process, as well as the possibility of genomic preservation by means of altered metabolic pathways. By quantifying and comparing inner $\Delta\psi M$ of the mutant *gas-1* to that of wild type animals, it will be possible to gain an understanding of the effects of the *gas-1* mutation on energy production in *C. elegans*. Because $\Delta\psi M$ drives production of ATP via OXPHOS, these data allow us to detect any change in reliance of *gas-1* on this pathway and whether a shift in metabolism may be providing genomic protection for the *gas-1* mutant.

Materials & Methods

Nematode culture conditions.

The *C. elegans* N2 strain and the nuclear mutant, *gas-1(fc21)*, were obtained from the Caenorhabditis Genetics Center (University of Minnesota). Prior to experimentation, the D. R. Denver lab (Oregon State University) generated a completely isogenic *gas-1* mutant strain via 10 generations of backcrossing to the N2 strain during which the *gas-1(fc21)* allele was tracked by PCR and Sanger DNA sequencing. Nematodes were thawed from a $-80^{\circ}\text{C}\pm 2^{\circ}\text{C}$ freeze and then kept on 60mm Petri plates containing 15mL of NGM-Lite agar that had been treated with 20 $\mu\text{g}/\text{mL}$ streptomycin and seeded with OP50-1 *Escherichia coli* as a food source. Worms were regularly transferred to new plates with the use of a sterilized platinum scalpel to maintain small population sizes, avoid starvation, and decrease risk of bacterial contamination. Strains were stored in a dark, 20°C incubator. Populations no older than 30 days were used in data collection.

Fluorescence microscopy.

All methods were adapted from Hicks et al. (2012). When a sufficient number of worms from both strains had reached adulthood, their eggs were harvested to ensure age-synchronicity during future data collection. Age-synchronization was achieved by repeatedly rinsing samples with M9 buffer solution, dissolving adult worms with a 2:1 NaOH:bleach solution, and frequent centrifugation to promote sedimentation of egg pellets (Hicks et al., 2012).

After the harvested eggs had matured on fresh plates for 24-58 hours, they were allowed to feed on fluorescently dyed *E. coli* for 24 hours (Hicks et al., 2012). A solution of 5 μ L of Chloromethyl-X-Rosamine (CMXROS) diluted with 495 μ L of M9 buffer solution was used per plate. CMXROS is a lipophilic, cationic, fluorescent dye that selectively binds to negatively charged components within the inner mitochondrial membranes of the worms (Pendergrass, Wolf, and Poot, 2004). The worms were then rinsed in M9 buffer solution and allowed to feed on un-dyed *E. coli* for 1 hour to pass any residual dyed *E. coli* from their intestinal tracts. Immediately prior to imaging, all worms were treated with a 20 mM solution of paralytic levamisole. The pharyngeal bulb of each worm was imaged at a total magnification of 25X, with the use of a fluorescence stereoscopic dissecting microscope (Leica MSV269). This neuromuscular organ was chosen to quantify $\Delta\psi_M$ because of the large amount of localized mitochondria typically found within the boundaries of the bulb (Hicks, et al. 2012). It is also a site that contains minimal amounts of auto-fluorescent fat deposits, which decreases the likelihood of fluorescent interference during imaging.

Image processing and data analysis.

After images were collected, each bulb was manually encapsulated using ImageJ software (NIH). They were then analyzed by measuring mean and maximum fluorescence values and total area of each bulb in ImageJ. Statistical comparison of the two strains was achieved using a non-parametric Wilcoxon chi-square approximation (first data set) or a Student's t-test (second data set) performed in JMP 9.0 (SAS). During image analysis, it became apparent that half of the nematodes comprising the first data set had not matured to the young adult stage prior to imaging. Because of this, a second set of data was collected and analyzed after the new sample had been verified to be young adult nematodes.

Results

Two sets of data from a total of three imaging sessions that took place in July and August were analyzed separately. Data were grouped in this fashion due to among-assay differences in nematode developmental stage (above). The first set of data (*Figure 1*) indicated that *gas-1* exhibited significantly lower levels of $\Delta\psi_M$ than the wildtype N2 strain (Wilcoxon $\chi^2 = 14.3$, $df = 1$, $N = 133$, $p = 0.0002$). Analysis of mean pixel intensity showed an identical pattern (data not shown). The second set of data (*Figure 2.1*) showed that *gas-1* trended toward higher $\Delta\psi_M$ levels than the wildtype N2 strain ($t = -1.44$, $df = 1$, $N = 77$, $p = 0.1547$), although the difference was not statistically significant. It was also observed that the wildtype strain had significantly larger pharyngeal bulbs than those of *gas-1* ($t = 6.06$, $df = 1$, $N = 77$, $p < 0.0001$) in the second data set (*Figure 2.2*).

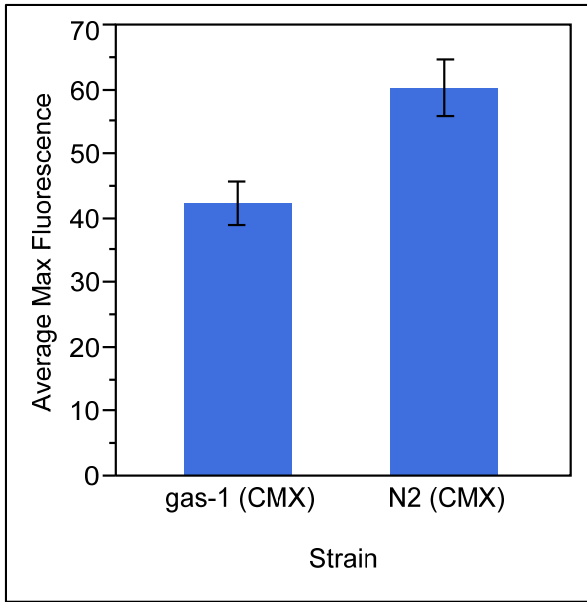


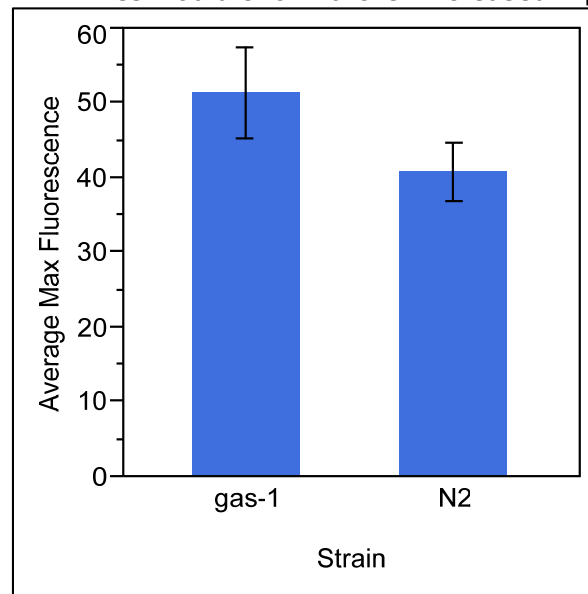
Figure 1: *gas-1* strain exhibits lower levels of average maximum fluorescence within pharyngeal bulbs stained with MitoTracker CMXROS. Maximum fluorescence is measured in pixel intensity, Y-axis. Error bars indicate one SEM (SD/\sqrt{N}).

Conclusion:

The first set of data ($N = 133$) indicated that *gas-1* exhibited lower $\Delta\psi_M$ than that of wildtype N2 (Figure 1), although upon closer inspection it was determined that the animals were not imaged at the appropriate young adult stage necessary for accurate data collection. As *C. elegans* reaches maturity, it experiences a marked increase in the numbers of mitochondria accompanied by a shift in primary metabolic reliance from the TCA cycle/glyoxylate cycle to OXPHOS (Intermediary metabolism: Metabolic patterns during development and aging: 7-9, Wormbook, 2009); therefore, a lack of metabolic maturity could underlie the lower $\Delta\psi_M$ of *gas-1* compared to the wildtype N2 strain. Results from the second set of data ($N = 77$), which used a verified young adult population, suggested that the *gas-1* population expressed higher levels of $\Delta\psi_M$ than that of the wildtype population (Figure 2.1), although the difference was not statistically significant. This observation could indicate that the *gas-1* mutation affecting ETC Complex 1 prohibits dissipation of $\Delta\psi_M$ down

the ETC, resulting in a highly polarized electrochemical gradient, which could also be contributing to *gas-1*'s characteristically high ROS levels and abnormally low level of ATP production (Brand, 2000). The second set of data also revealed that *gas-1* had significantly smaller mean pharyngeal bulb areas than those of N2 (Figure 2.2). This large difference in bulb size between strains could be explained by the less efficient and less healthy metabolism of *gas-1*. Similarly, it is well-understood that the movement of the pharynx, or pharyngeal pumping, is recognized as a proxy for metabolic rate in *C. elegans* (Collins, 2008).

Future work will conduct additional trials using a larger sample size will be used during data collection, and use confocal fluorescence microscopy, which will allow imaging of separate focal planes at higher magnification and increased resolution. This experiment will also be repeated using ten mutation-accumulation (MA) lines generated from either *gas-1* or N2 (in addition to the *gas-1* and N2 progenitor strains) to further explore the possibility of a shift in metabolism from OXPHOS to glycolysis among lines that have accumulate mutations under genetic drift against either the *gas-1* mutant or wildtype genetic background. Based on my preliminary findings, I would expect that *gas-1* MA lines would show further increased $\Delta\psi_M$



levels and would show greater variability in $\Delta\psi_M$ levels from *gas-1* progenitor than N2 MA lines would show compared to N2 progenitor. In contrast, I would expect N2 MA lines to

have similar or lower levels of $\Delta\psi_M$ compared to N2 progenitor.

Figure 2.1: *gas-1* strain exhibits higher average maximum fluorescence within pharyngeal bulbs stained with MitoTracker CMXROS than that of wildtype. Maximum fluorescence is measured in units of pixel intensity, Y-axis. Error bars indicate one SEM (SD/\sqrt{N}).

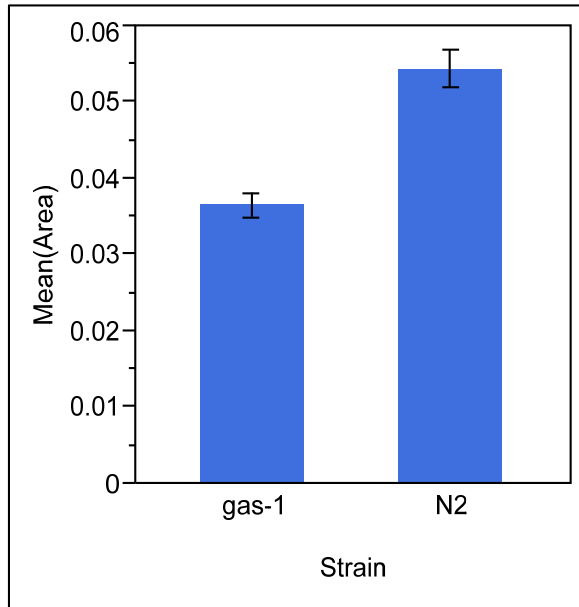


Figure 2.2: *gas-1* strain exhibits smaller pharyngeal bulb area than that of wildtype N2 when CMXROS stained organs are manually encapsulated and measured with ImageJ data analysis software. Pharynx area is measured in pixels², Y-axis. Error bars indicate one SEM (SD/\sqrt{N}).

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