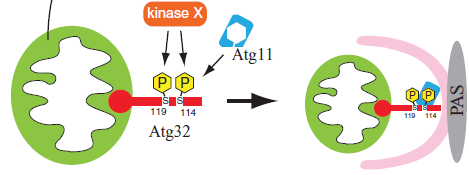
**The Effects of Increased Net Reactive Oxygen Species on Mitophagy**

1. **Introduction**

As the global human population continues to age and live longer lives, both developed and developing countries have begun to feel the negative impact of neurological disorders. According to the National Institute of Health, 1 in 7 people in the United States will develop dementia or some form of neurodegenerative disease once they reach 70 years old. Therefore, there has been a growing interest in this field. These disorders are an epidemic that ignore race, age, education, income, and sex.

Reddy et al (2013) speculated that alcohol-induced oxidative stress can alter the brain mitochondria. Reactive oxygen species (ROS) are natural by-products of mitochondria in cells when producing energy. ROS can damage proteins, mitochondria, and DNA. If these damaged structures are not disposed of, they can accumulate and cause cellular dysfunction or even death. The key feature of neurodegeneration is the lack of dysfunctional mitochondria being disposed of properly.

Cells use autophagy to prevent this build-up of dysfunctional organelles. This is done by signaling the dysfunctional structures for regulated destruction by lysosomes, which contain an acid enzyme that breaks down the materials. A more selective form of autophagy is mitophagy, which is the breakdown of dysfunctional mitochondria. The mechanism of mitophagy is still not quite understood. In mammals, the proteins identified in regulating this process are called PARK1 and PARK4. In yeast cells, the regulatory proteins are called Atg-32 and Atg-11 (Aoki et al 2011).

**Figure 1: Overview of Mitophagy Mechanism. Adapted from Figure 6 of Ref Aoki et al 2011**

Atg-32 and Atg-11 are specific proteins located in the cytoplasm that have been identified to regulate mitophagy in *saccharomyces cerevisiae* (yeast). When a dysfunctional mitochondria is detected, Atg-32 attaches itself on the outer mitochondrial membrane. When the Atg-32 protein is phosphorylated by an unknown kinase, it develops a high affinity to Atg-11. Atg-11 is a protein that initiates the phagophore assembly site (PAS) where cytosolic membrane vesicles are formed. Atg-11 then interacts with the mitochondrial residential receptor Atg-32, forming a mitochondrial-protein complex. The autophagosome membrane forms around the complex and then proceeds to fuse with a lysosome where the cargo (mitochondria) is then degraded. The overview of this process is in Figure 1 (Aoki et al 2011).

However, this act of balancing the levels of ROS is not perfect. Outside factors could contribute and overwhelm the system. Lin et al (2006) speculated that an excess of ROS in a cell can damage mitochondria and cause a build-up of these dysfunctional structures. This imbalance of ROS in a cell can cause a phenomenon called “net ROS” and is thought to be the main contributor to aging, which is a major risk factor for dementia. When there is not an even ratio of ROS being produced and removed, there can be a surplus of ROS in the cell. The build-up of these dysfunctional organelles can cause cell death. There is evidence that people with long-term alcohol abuse use have higher chances of developing neurodegenerative disease in old age compared to people who drink more casually or occasionally (Lin et al 2006). This could be because the excess ROS inhibits the proper regulation of dysfunctional mitochondria.

The purpose of the experiment described in this proposal is to determine if higher concentrations of ethanol will show low levels of GFP processing, which correlates to lower levels of mitophagy, in yeast (Aoki et al 2011).

1. **Experiment**

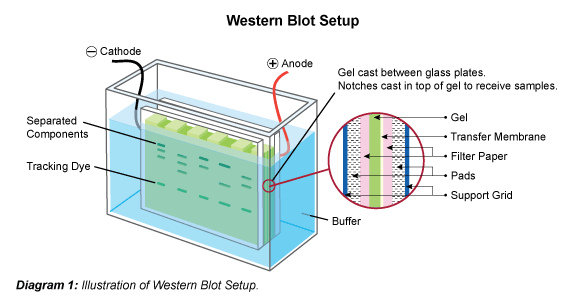
The aim of this experiment is to determine if higher concentrations of ethanol exposure will decrease levels of Om45-GFP processing by yeast cells in response to ethanol. If the phosphorylation of Atg-32 is necessary for mitophagy, then I would expect that higher net levels of ROS would decrease levels of GFP processed because the kinase processing Atg-32 has a higher chance of being oxidized and becoming dysfunctional. The correlation of GFP processing and levels of mitophagy is described in section II.A.

I will produce Atg-32 protein expressing Om45-GFP in yeast by using a viral vector. This technique is done by exposing the virus carrying the gene for Atg-32 expressing Om45-GFP with the yeast cell in order to transfer the gene directly. I will introduce the yeast cell groups to different levels of ethanol as their environment. My control group will be yeast cells not in ethanol. The levels of mitophagy will be correlated by the amount of GFP processed using a Om45-GFP processing assay and a western blot technique as described by Aoki et al (2011) and in II.A/II.B.

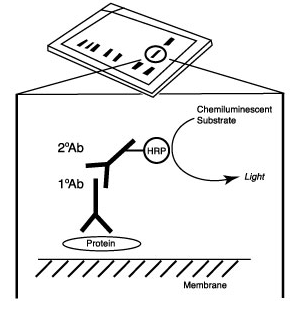
II.A Measuring Levels of Mitophagy in Yeast

This method was employed by Aoki et al (2011) to detect for the evidence of changing rates of mitophagy in yeast induced by nitrogen starvation. Aoki et al (2011) speculated that the phosphorylation of Ser-114, a specific amino acid structure on Atg-32, might be important for mitophagy and for the Atg-32 affinity for Atg-11. They tested this idea by producing phosphorylation-deficient Atg-32 mutants expressing Om45-green fluorescent protein(GFP) and inducing the yeast cells to undergo mitophagy by nitrogen starvation.

Om45 is a mitochondrial outer membrane protein that has no known function. When Om45 is tagged with GFP, the complex attaches itself around the mitochondrial outer membrane. Each group of mutants had a different amino acid that was phosphorylation-deficient on the Atg-32 protein. One group was the wild-type, one group was deficient in Ser-114, one group was deficient in the Ser-119, and one group was deficient in both Ser-114 and Ser-119.

When the yeast cells underwent mitophagy, Om45-GFP ends up at the cell’s vacuole after the mitochondria is degraded by the lysosome. The Om45-GFP complex is then broken down. GFP is stable within the vacuole, so it is released as an in-tact protein. The level of GFP processed is correlated to the level of mitophagy that the cell underwent. The way that Aoki et al measured the level of GFP is explained in II.B.

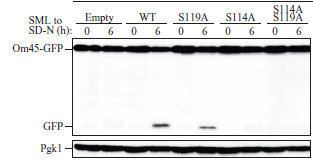
II.B Western Blot

Western blotting is an analytical technique that is used to detect for specific proteins in a given sample. An overview of the set-up for Western Blot can be seen in Figure 2. After the induction of mitophagy, the yeast cells are lysed and the contents of each of the individual cells are then collected. The samples are then put on a gel and electricity is run through the gel, which separates the proteins based on size (gel electrophoresis). After the proteins are separated, they then have to be transferred over to a membrane and sandwiched like in the diagram in Figure 2. The empty nodes in the membrane have to be blocked with casein, a protein buffer that stops antibodies from attaching. When the current is run through again, the proteins will move onto the membrane in the same order that they were in the gel.

**Figure 3: Primary antigen and Secondary antigen binding on a specific target protein on the membrane. This is pulled from reference #5**

**Figure 2: Set-up of the western blot as described in section II.B. Membrane is sandwiched between the gel, filter paper, pads, and support grid.**

Aoki et al used a primary antibody called anti-GFP in order to detect the specific protein being monitored. The antibodies will attach themselves to any GFP protein on the membrane. A second dyed anti-body is then used that will attach to the primary antibody. An example of how this is done is shown in Figure 3.The amount of GFP processed was then detected as bands that migrated from the Om45-GFP band. Only properly processed GFP migrated from the Om45-GFP band. By measuring the levels of GFP processed from Om45-GFP, Aoki et al was able to correlate that measurement to levels of mitophagy in the yeast cell.

What Aoki et al (2011) observed was that the wild-type yeast cells had a darker band in the gel, which indicated high levels of GFP protein in-tact. They observed a lighter band in the gel for Ser-119 deficient, which meant less GFP processed compared to the wild-type. The yeast cells with Ser-114 deficiency and with both Ser-114 and Ser-119 deficiency had no band shown, indicating that none to very little GFP was in-tact. These bands corresponding to each group can be all seen in Figure 4.

**Figure 4. Western blot of the results from the Om45-GFP Processing Assay performed by Aoki et al. The dark bands represent the number of GFP in-tact, which correlates to levels of mitophagy in cell. The darker the band, the higher level of activity.**

1. **Discussion**

If all goes well and my predictions are correct, high levels of net oxidative stress due to ethanol exposure will cause a decrease levels of GFP processing, which could mean that there is a decrease in levels of mitophagy. This data could be used to provide a greater link between the development of dementia and long-term alcohol consumption.

The biggest problem behind this experiment is that there is very little knowledge about the specific mechanisms of mitophagy. What we have learned so far about the proteins responsible were only very recent findings. The identity of the kinase (only designated as X in Figure 1) that phosphorylates Ser-114 has not been identified yet, but we just know that this event happens and that it is necessary for the initiation of mitophagy.

There will be great difficulty in actually interpreting the results because of this unknown kinase. Even if we are able to determine if GFP processing decreases, we do not have the knowledge yet to determine to what extent the effects of the decreased levels of mitophagy actually stems from. There could also be many other factors as a result of using ethanol. Free oxidative species are not the only results of ethanol exposure.

Neurological disorders such as dementia are difficult to diagnose and treat because much of human brain function is still widely unknown. If the results of this experiment suggest that increasing levels of net ROS can increase the risk for developing dementia later in life for a human being, then physicians can work their way towards providing more options to diagnose and prevent the disorder. The science community will also make progress towards increasing the understanding of mitophagy and how oxidative stress affects the mechanism.

**References**

[1] Almansa, I., A. Fernandez, C. Garcia-Ruiz, M. Muriach, J. Barcia, M. Maranda, J. Fernandez- Checa, F. Romero. 2009. Brain mitochondrial alterations after chronic alcohol consumption. The Journal of Physiology and Biochemistry 65: 305-312

[2] Aoki, Y., T. Kanki, Y. Hirota, Y. Kurihara, T. Saigusa, T. Uchiumi, D. Kang. 2011. Phosphorylation Serine 114 on Atg32 mediates mitophagy. The Molecular Biology of the Cell 22: 3206-3217

[3] Ashrafi, G., T. Schwarz. 2013. The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death and Differentiation 20: 31-42

[4] Bailey, S. M. 2003. A Review of the Role of Reactive Oxygen and Nitrogen Species in Alcohol-induced Mitochondrial Dysfunction. Free Radical Research 6: 585-596

[5] Cell Signalling Technology Inc. “Western Blotting” Accessed December 7, 2013. http://www.cellsignal.com/products/7074.html

[6] Dolganiuc, A., P. Thomes, W. Ding, J. Lemasters, T. Donuhue Jr. 2012. Autophagy in Alcohol-Induced Liver Diseases. Alcoholism: Clinical and Experimental Research 36: 1301-1308

[7] Kanki, T., D. Kang, D. Klionsky. 2009. Monitory mitophagy in yeast. Autophagy 8: 1186- 1189

[8] Knorre, D., K. Popadin, S. Sokolov, F. Severin. 2013. Roles of Mitochondrial Dynamics under Stressful and Normal Conditions in Yeast Cells. Oxidative Medicine and Cellular Longevity 1: 1-6

[9] Kondo-Okamoto, N., N. Noda, S. Suzuki, H. Nakatogawa, I. Takahashi, M. Matsunami, A. Hashimoto, F. Inagaki, Y. Ohsumi, K. Okamoto. 2012. Autophagy-related Protein 32 Acts as Autophagic Degron and Directly Initiates Mitophagy. The Journal of Biological Chemistry 287: 10631-10638

[10] Kurihara, Y., T. Kanki, Y. Aoki, Y. Hirota, T. Saigusa, T. Uchiumi, D. Kang. 2012. Mitophagy Plays an Essential Role in Reducing Mitochondrial Production of Reactive Oxygen Species and Mutation of Mitochondrial DNA by Maintaining Mitochondrial Quantity and Quality in Yeast. The Journal of Biological Chemistry 287: 3265-3272

[11] Leinco. "Western Blot Setup." *Leinco*. Accessed December 7, 2013. http://www.leinco.com/general\_wb

[12] Lin, M., M. Beal. 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443: 787-795

[13] Okamoto, K., N. Kondo-Okamoto, Y. Ohsumi. 2009. A landmark protein essential for mitophagy. Autophagy 5: 1203-1205

[14] Reddy, V., P. Padmavathi, G. Kavitha, B. Saradamma, N. Vaaradacharyulu. 2013. Alcohol- induced oxidative/nitrosative stress alters brain mitochondrial membrane properties. Molecular Cell Biochemistry 375: 39-47

[15] Schreiner, B., H. Westerburg, I. Forne, A. Imhof, W. Neupert, D. Mokranjac. Role of the AAA protease Yme1 in folding of proteins in the intermembrane space of mitochondria. Molecular Biology of the Cell 23: 4335-4346

[16] Wager, K., C. Russell. 2013. Mitophagy and neurodegenernation: The zebrafish model system. Autophagy 9: 1-17

[17] Wang, K., M. Jin, X. Liu, D. Klionsky. 2013. Proteolytic processing of Atg32 by the mitochondrial i-AAA protease Yme1 regulates mitophagy. Autophagy 9: 1-9