SAUTERER LAB RESEARCH INTERESTS:

INTRODUCTION AND LITERATURE REVIEW:

Apoptosis is the controlled process of cell suicide, which is vital in development and body maintenance. During apoptosis, caspases and nuclease enzymes are activated which destroys cellular proteins, DNA, and ultimately destroys the cell (Lockshin, et al 1998, Lodish, et al, 2012). Examples of apoptosis include the monthly menstrual flow, the loss of tissue around developing fingers, and resorption of tadpole tails during metamorphosis. Although apoptosis can be initiated by numerous triggers, a major inducer of apoptosis is mitochondrial damage that results in leakage of mitochondrial proteins into the cytoplasm. The mitochondrial protein cytochrome *C* is especially potent promoters of apoptosis.

DNA damage also can initiate apoptosis. DNA or chromosomal damage alters chromatin structure and releases the chromosomal histone proteins into the cytoplasm. Histones are the major proteins of chromatin in eukaryotes and are found in some Archeans as well. The basic structure of chromatin is the nucleosome, where DNA wraps around a core of two copies each of histones H2A, H2B, H3 and H4. A linker histone, H1, joins nucleosomes together to form higher-order structures such as the 30 nm fiber that is the basic structural element of chromosomes (Weaver, 2012 Lodish et al, 2012, Darnell, 2012). Additionally, histones inhibit transcription by binding to DNA so tightly that the proteins needed to activate transcription. Histones can be epigenetically modified by covalent attachment of phosphates, acetyl groups, methyl groups and small proteins (SUMO and ubiquitin) to regulate their attachment to DNA and serve as binding sites to chromatin remodeling complex proteins and transcription factors (Darnell, 2012). For example, histone phosphorylation is required to loosen their binding to DNA so they can be displaced from critical DNA sequences that are required for transcription. Other histone modifications can cause long-term epigenetic changes that affect transcription of specific genes (Darnell, 2012).

One mechanism that induces apoptosis after DNA damage was demonstrated by Cascone et al (2012). Using rat liver cells, they demonstrated that the core histones (H2A, H2B, H3 and H4) and the linker histone (H1), bind specifically to mitochondria and induce permeabilization of the mitochondrial outer membrane to cause the release of the cytochrome *C* which induces apoptosis. In addition, the linker histone H1 binds to mitochondria and causes permeabilization of ***both*** the mitochondrial inner and outer membranes, resulting in loss of the H+ gradient that powers ATP production (Cascone et al 2012). The histones interact with the mitochondrial outer membrane to induce the formation of pores in the membrane that allows the contents to leak out. Our previous work, which formed the core of Linda Major's master's thesis (Major, 2014), showed evidence that plant histones bind to plant mitochondria and induce release of cytochrome *C* from the mitochondria, but that histones do not bind to plant chloroplasts. This data suggests that the mitochondrial-histone interaction and effects is conserved across two divergent eukaryotic kingdoms. However, resource and time limitations allowed us to investigate histone-mitochondria binding using antibodies only against a single core histone, H3, so it is still unknown whether any of the other histones bind to mitochondria. Furthermore, the salt-extracted histone fractions (Shechter et al 2007) were only about 50% histones as determined by electrophoresis and contained numerous other proteins, so our data to date only confirms that histone H3 binds to mitochondria and that *something* in the histone fractions, though perhaps not the histones themselves, causes cytochrome *C* release from mitochondria. Similar investigations using cultured yeast are in the early stages (see progress report).

In order to refine these preliminary studies and make them publishable, we propose to obtain *highly purified* histone fractions, using histones purified using commercial histone purification kits and/or purification of the crude histone fractions using HPLC chromatography (Shechter et al 2007) using the HPLC system in the Chemistry Department and with the assistance of Dr. Nixon Mwebi. High-pressure liquid chromatography or HPLC utilizes chromatography columns with microscopic particle size, giving high binding capacity and exceptional resolution, but requiring extremely high pressures (roughly 100 atmospheres) to force liquids through the column. HPLC offers the potential to purify each individual histone from a partly purified mixture, so that the interaction of each histone on mitochondria can be examined individually. Additionally, we will investigate histone-mitochondrial binding using both our histone H3 antibodies and a second core histone antibody (H2A) purchased from this grant. We will use histones and mitochondria purified from cauliflower (Major, 2014) and/or yeast to perform these investigations. If time and resources permit, we will also perform cross-incubation studies (histones from one organism with mitochondria from another) to determine the conservation of the histone-mitochondrial interaction. The proposed investigations will help determine if histone-mitochondrial interactions are broadly conserved across multicellular eukaryotes, and could be expanded to investigate other eukaryotes such as various protest kingdoms in future studies.

Methods:

1. Model organism and cell fractionation:

Two model organisms will be used: cauliflower curds (Major, 2014) and yeast. Fresh store-bought cauliflower will be prepared according to Major (2014). Briefly, cauliflower curds are cut up into small pieces, then ground in a mini-Ninja blender. Larger pieces and intact cells are removed by centrifugation, and crude nuclei and mitochondria are obtained by differential centrifugation. The nuclei and/or mitochondria are further purified by Percoll density gradient centrifugation (Major 2014). These procedures give variable yields, however, due to the thick cell walls of cauliflower cells. An alternate purification method, which preliminary studies showed promise (Major, 2014) but were not pursued further due to budgetary limitations, is to remove the cell walls using commercial enzyme mixtures (Viscozyme), generating cell wall-free protoplasts. Protoplasts can then be used to obtain organelles by differential and density gradient centrifugation.

We will culture common baker's yeast in 2 liter sterile flasks on a rotary shaker (120 RPM at 37^{o}C) in a medium containing glucose, peptones, yeast extract, and minerals (Xiao, 2006 for different culture protocols). Cells will be harvested by centrifugation when they grow to an optical density (600 nm) of 0.5 – 1.0, indicating log-phase growth of a healthy culture. In order to isolate yeast organelles such as nuclei and mitochondria, cell walls must be removed to obtain yeast protoplasts (spheroplasts) by enzymatic digestion of the cell walls using the commercial enzyme mixture, Lyticase (Sigma) (Clarke, et al, 1993). The progress of the spheroplasting process is verified by observing the decrease in absorbance at 600 and 800 nm as the cell walls are dissolved. Studies determining optimal culturing and spheroplasting conditions are in progress.

2. Mitochondrial isolation:

Mitochondria will be purified from cauliflower fractions and yeast spheroplasts by a combination of differential centrifugation and density gradient centrifugation. Cauliflower mitochondria will be isolated by two (low and high-speed) centrifugations, and the resulting pellet further fractionated by centrifugation, then the crude mitochondrial pellet will be fractionated on a 20%-33%-80% Percoll density gradient. Mitochondria settle at the 20%-33% interface and are collected and washed (Major, 2014). Yeast mitochondria will be obtained from protoplasts based on the methods of Meisenger et al (2006), Gregg et al (2009) and Patel (2014) and will be modified as required. Yeast protoplasts are homogenized with a Teflon homogenizer in a buffer containing 0.6 M mannitol or sorbitol, EDTA, BSA and protease inhibitors. Intact cells and debris are removed by centrifugation (1500 xg for 5 min), then the supernatant is centrifuged at high speed to get a crude mitochondria pellet. Mitochondria are further purified on a 15%, 23% and 40% Percoll density gradient. The resulting mitochondria should be highly pure and lacking nearly all other contaminating organelles. Mitochondria will be observed microscopically and tested for viability by adding 0.1 mM DCIP, which absorbs electrons released during photosynthesis or oxidative phosphorylation, turning blue to colorless in the process. Pelleted mitochondria can be frozen at – 80 ^{0}C.

3. Purification of crude histones:

Crude cauliflower nuclei are obtained by differential centrifugation (Major, 2014). Yeast nuclei can be purified from spheroplasts by several methods (Hahn, 2006, Zhang and Reese, 2006, Lowary and Widom, 1989). Spheroplasts are lysed in a Teflon homogenizer in a buffer containing 18% Ficoll (Zhang and Reese, 2006, Lowary and Widom, 1989) or a glycerol-detergent buffer (Hahn, 2006). Nuclei from lysed cells are prepared by differential centrifugation steps to remove unwanted debris (Hahn, 2006) or step gradient centrifugation using Ficoll and/or sucrose step gradients (Zhang and Reese, 2006, Lowary and Widom, 1989). Nuclei are examined for purity under a phase-contrast microscope and by Hoechst staining (a DNA-specific fluorescent stain). Crude histones from nuclei can be isolated either by acid extraction or by high-salt extraction. To isolate histones, the acid extraction procedure (Schechter et al 2007, Brandt et al 1980, Clarke et al, 1993) involves incubating nuclei in 0.2 – 0.4M H_2SO_4, which precipitates nearly all proteins and DNA but dissolves the histones, then centrifuging precipitated material and precipitating the histones from the supernatant by cold ethanol or trichloroacetic acid (TCA). The high-salt extraction procedure (Schechter, et al 2011) involves lysing the nuclei in a salt-free buffer, centrifuging down the chromatin and nuclear fragments, then extracting the histones in a buffer containing 2.5 M NaCl, which dissolves the histones. The sample is centrifuged to remove DNA and insoluble debris and histones in the supernatant are

precipitated as described above. Histone samples will be examined for purity by SDS-PAGE and immunoblotting with anti-histone antibodies (below).

4. Highly purified histones:

Crude histones must be further purified in order to eliminate the possibility that contaminating proteins are actually what induced mitochondrial cytochrome *C* release. Companies such as Epigentek and Invent Biotechnologies offer histone purification kits, using proprietary resins and elution buffers, that can isolate core (but not H1) histones from crude cell extracts to near 90% purity and higher purification if using crude histone extracts as previously described. These kits will be used to obtain highly purified histones that can either be used directly for incubation experiments (Section VI) or for HPLC purification of individual histones. Two different kits, using different histone purification methods, will be tested and the best used for subsequent experiments. The purity of the histones in each sample will be assessed by SDS-PAGE (14% gels) and by immunoblotting against one of the core histones.

5. HPLC purification of histones:

We will attempt the purification of the individual hist**o**nes (the core histones and H1) by HPLC initially from one of the model organisms. HPLC purification will be performed in collaboration with Dr. Mwebi, (JSU Chemistry Department) who will teach us how to use the system, the theory behind it and assist in the analysis of the fractions. HPLC purification of histones will be based on the method of Shechter et al (2007). Briefly, partly purified histones will be filtered, injected into the HPLC and separated on a C8 (Shechter, et al 2007) column (JSU Chemistry Department), which binds hydrophobic molecules, and fractionated using a gradient elution of acetonitrile and acetonitrile - 0.1% trifluoroacetic acid. Initial runs using purified commercially available mammalian histones will be performed under similar conditions to determine location of the histone peaks. Histone-containing fractions will be collected, analyzed by SDS-PAGE and Western blotting against the appropriate histone antibody. Histone fractions will then be lyophilized and stored at -80^0C.

6. Testing mitochondria for histone binding:

Testing for histone binding to organelles will be based on the method of Cascone et al (2012) as performed by Major (2014). Histones at 1 uM (20 ug/ml) will be added to 0.5 mg/ml mitochondria in a physiological buffer for 10 minutes, then centrifuged 5 minutes at 10,000 xg. Mitochondria will be incubated with 1 mM succinate as an energy source. The mitochondrial pellet and supernatants will be analyzed by SDS-PAGE and immunoblotting using anti-H3 and H2A histone antibodies, and anti-cytochrome *C* antibodies. Supernatants may be concentrated by TCA precipitation or by use of centrifuge concentrators to provide a high concentration of protein for detection. Control experiments will involve incubation of organelles with 1 uM (60 ug/ml) BSA. If histones do specifically bind to mitochondria, the mitochondrial pellets should show large amounts of histones while little should be found in the supernatants. Control experiments should show no histones in the mitochondrial pellets since none were added. If time and resources permit, cross-incubation studies, such as incubation of yeast histones with cauliflower mitochondria or vice versa, will be performed as well.

7. Detection of mitochondrial permeabilization:

Mitochondria will be incubated with histones or control BSA as previously described, then pellets and supernatants will be collected. Mitochondrial pellets and concentrated supernatants will be analyzed by immunoblotting using anti-cytochrome *C* antibodies. Detection of cytochrome *C* in the experimental supernatant and no detection in the control supernatant will indicate that histones permeabilize the mitochondria and allow cytochrome *C*, a major apoptosis promoter, to leak out. Supernatants may be concentrated by centrifuge concentrator devices or TCA precipitation as the detected antibody signal in unconcentrated supernatants is very low (Major, 2014).

8. SDS-PAGE and Immunoblotting:

Samples will be analyzed by SDS-PAGE on 14% gels by standard procedures. Immunoblotting will be performed by transferring proteins from SDS-PAGE gels by electrophoresis onto nitrocellulose membrane, then immunoblotted using primary (antibodies to histones and cytochrome *C*) antibodies and alkaline phosphatase–coupled secondary antibodies according to manufacturer's directions, since each antibody requires specific dilutions and some require special washing procedures. Positive controls of cytochrome *C* and histones will be separated on SDS-PAGE gel lanes with each experiment to insure that the immunoblotting procedures do indeed detect these proteins.