5-2014

MitoNEET: Reduction in Insulin Resistance through Ameliorated Oxidative Stress?

Nicolas A. Ferry

MitoNEET: Reduction in Insulin Resistance through Ameliorated Oxidative Stress?

by

Nicolas A. Ferry

HONORS THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE IN BIOLOGICAL SCIENCES WITH HONORS

AT EASTERN ILLINOIS UNIVERSITY

CHARLESTON, ILLINOIS

May 2014

I hereby recommend that this Honors Thesis be accepted as fulfilling this part of the undergraduate degree cited above:

Thesis Director	05/01/14 Date
Honors Program Director	5/1/2014 Date

Abstract

MitoNEET is a mitochondrial iron-sulfur protein with poorly-understood functions. Proposed functions of mitoNEET include regulating of oxidative capacity, reactive oxygen homeostasis, and other possible roles. Investigating the interactions of mitoNEET with other proteins might help identify functional roles of this protein. MitoNEET was discovered in 2004 to identify physiological targets of pioglitazone hydrochloride, a drug that is commonly used to treat type-2 diabetes. Type-2 diabetes is a metabolic disease and, although the exact pathology is complex, the role of the mitochondrion in cellular energy production and glucose oxidation indicates that this organelle has a role in type-2 diabetes. Because of the specific location of mitoNEET and its interaction with pioglitazone, there is the potential for this protein to have a role in diabetes treatment and/or prevention. My protein pull-down experiments indicated a direct interaction between mitoNEET and the enzyme catalase, which decomposes hydrogen peroxide (H_2O_2). Ultraviolet-visible spectroscopy of catalase, before and after the addition of mitoNEET, showed changes in absorbance at λ = 270, 320 and 400 nm, indicating an interaction between the proteins. The rate of catalase activity was measured by following the breakdown of H_2O_2 over time. The average rate of catalase activity in absence of mitoNEET was 0.0899 ± 0.0195 nM H_2O_2 min^{-1} μg $protein^{-1}$ and this rate increased after addition of mitoNEET to 0.0126 ± 0.0253 nM H_2O_2 min^{-1} μg $protein^{-1}$. My findings may indicate that mitoNEET decreases cellular H_2O_2 levels through activation of catalase, and thereby reduces the probability to develop oxidative stress induced insulin resistance.

Acknowledgments

I would like to thank my research mentor Dr. Michael Menze for giving me the opportunity to research in his laboratory as well as teaching me the essential skills required to perform these experiments. I would also like to thank Dr. Mary Konkle for her time and expertise that also made my project a possibility. I am eternally grateful for all of the experience and knowledge I have gained from this opportunity to take part in undergraduate research. Additionally, I would like to thank the Eastern Illinois University Honors College for the Undergraduate Research Scholarship and Creative Activity Grant, which allowed me to remain in Charleston and perform research over the summer. Finally, I would like to extend my deepest gratitude to the entire EIU Department of Biological Sciences faculty and staff for challenging me to grow in the classroom and providing an amazing experience at EIU.

Table of Contents

Introduction

MitoNEET, also known as CISD1 (GenBank accession number: Q9NZ45.1), is a mitochondrial protein named for its location ("mito") and the presence of the amino acid sequence Asn-Glu-Glu-Thr (NEET; Paddock et al., 2007). MitoNEET was discovered in 2004 using a photoprobe of thiazolidinedione (TZD) in order to identify physiological targets of pioglitazone hydrochloride (Colca et al., 2004). Pioglitazone hydrochloride, marketed as Actos, is a drug that is commonly used to treat type-2 diabetes.

MitoNEET is primarily found in the outer membrane of the mitochondria, but small amounts of the protein may also distribute to the inner membrane facing the mitochondrial matrix (Paddock et al., 2007). MitoNEET is a small protein with a molecular mass < 17 kDa (Figure 1; Colca et al., 2004), and contains a transmembrane domain that anchors the protein to intercellular membranes (Wiley et al., 2006). The specific physiological role(s) of mitoNEET are still unclear although several functions have been proposed (Bak and Elliott, 2013). These possible functions of mitoNEET include acting as a redox sensor (Zhou et al., 2010; Landry and Ding, 2014), sulfur biogenesis (Zuris et al., 2011), a role in iron trafficking and metabolism (Kusminski et al., 2012; Nechushtai et al., 2012), and reactive oxygen homeostasis and regulation of oxidative capacity (Wiley et al., 2006; Tsai et al., 2009). Other possible roles include a role in signaling of apoptosis (Chang et al., 2009; Chen et al., 2010; Maiuri et al., 2010), and a role in 2Fe-2S cluster

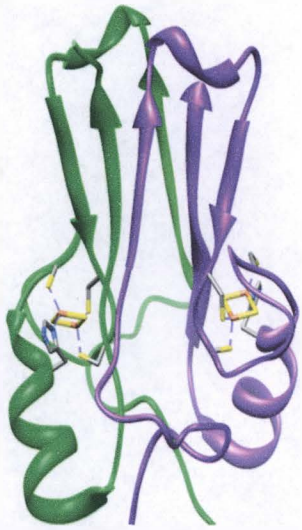

Figure 1. Crystal structure of mitoNEET homodimer (Protein Data Bank [PDB] 4F28).

transfer to target proteins (Paddock et al., 2007; Conlan et al., 2009b; Zuris et al, 2011; Baxter et al., 2012). Another study has theorized mitoNEET to alter mitochondrial oxidative capacity (Geldenhuys et al., 2011). Investigating the interactions of mitoNEET with other proteins might help to shed light on this protein. MitoNEET, a homodimer (Arif et al., 2011), is a metalloprotein (MP) with iron-binding properties (Lin et al., 2007) and belongs to a unique family of redox active 2Fe-2S proteins (Conlan et al., 2009b).

MPs are broken down into groups classified by their inorganic functional groups. MitoNEET is part of the iron-sulfur group, the largest grouping of MPs that contains a number of proteins that play roles in human health and disease (Napier et al., 2005). MitoNEET is further categorized into, and was the first protein discovered of, the "NEET" family which also contains the two proteins miner 1 (CISD 2, GenBank accession number: Q8N5K1.1) and miner 2 (CISD3, GenBank accession number: P0C7P0.1) (Nechushtai et al., 2012). While mitoNEET was initially discovered the relationship it shares with type-2 diabetes, miner 1 has recently been identified as one of the causative agents in Wolfram Syndrome (Conlan et al., 2009a; Rigoli and Di Bella, 2012). The NEET family ligates their clusters (Figure 2) with a unique 3-Cys-1-His coordination (Wiley et al., 2007). The cluster is the most stable at pH = 8.5, but is easily lost if pH < 7.0 (Dicus et al., 2010). The cluster is also more stable when the ligated iron is in the reduced state (Fe^{2+} vs. Fe^{3+}; Bak and Elliot, 2013). MitoNEET is also the first FeS protein known to bind to drugs (Colca et al., 2004).

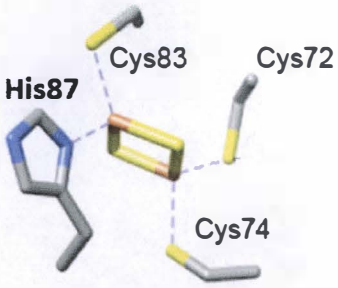

Figure 2. 3-Cys-1-His cluster ligation.

Type-2 diabetes, often associated with a sedentary life style, is a metabolic disease caused by the imbalance of chemical reactions in the body that sustain life (Patti and Covera, 2010). The disease has also been associated with high-fat diets (Anderson et al., 2009). The Centers for Disease Control and Prevention (CDC; 2014) reported that individuals diagnosed with diabetes had twice the risk of death compared to individuals of similar age not diagnosed with diabetes and the annual cost of treatment and other expenses was $174 billion. The number of newly diagnosed diabetes cases each year has risen above 1,600,000 (Figure 3).

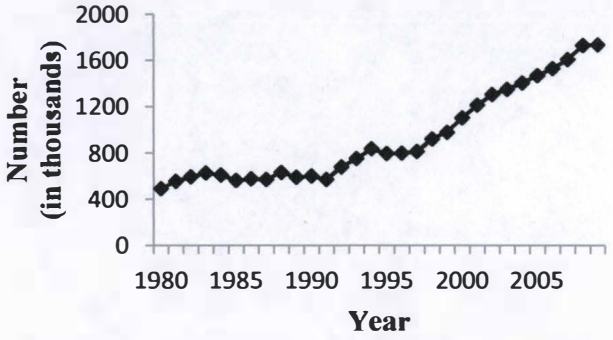

Figure 3. New cases of diagnosed diabetes among U.S. adults aged 18-79 years (CDC).

Although the exact pathology of type-2 diabetes is complex, the role of the mitochondrion in cellular energy production and glucose oxidation suggests that this organelle is crucial to the pathology of the disease (Wiley et al., 2006; Patti and Covera, 2010). The mitochondria in beta cells have even been found to supply specific metabolites, including malate, citrate, α-ketogluterate and succinate, during periods of elevated plasma glucose levels to support insulin secretion, (Jitrapakdee et al., 2010; Supale et al., 2012). Anderson et al. (2009) showed that high-fat diets, a common cause of type-2 diabetes, increased H_2O_2 production by the mitochondria. It is well known that type-2 diabetes initially causes improper use of insulin, and eventually leads to the inability of the body to produce insulin, or to respond to insulin (insulin resistance; CDC,

2014). This improper use includes defects in beta cell production and secretion of insulin, or the ability of the body to effectively utilize the polypeptide (Wiederkehr and Wollheim, 2006; National Diabetes Education Program [NDEP], 2011).

Past studies have shown that mitoNEET interacts with other molecules including resveratrol-3-sulfate, an intermediate in resveratrol catabolism in humans, and nicotinamide adenine dinucleotide phosphate (NADP(H)), a coenzyme that participates in electron transfer reactions in many metabolic processes (Zhou et al., 2010; Arif et al., 2011). Resveratrol treatment has been shown to normalize hyperglycemia and hyperinsulinemia (Ramadori et al., 2009). To identify other possible binding partners of mitoNEET, and to further investigate the properties of mitoNEET, an associated research lab performed a protein pull-down experiment, the results of which identified several possible protein binding partners, one being the enzyme catalase (M.E. Konkle, personal communication).

Catalase was first crystalized in 1937 (Zamocky and Koller, 1999), and is a homotetramer (Figure 4) with a subunit mass of 60 kDa (Kirkman and Gaetani, 2006). Catalase, found in almost every living organism (Loew, 1900), is located in several cellular compartments, including the mitochondrion (Nicholls, 2012). A previous study showed that catalase overexpression resulted in a 20% increase in the lifespan of mice (Schriner et al., 2005). Catalase detoxifies the cell through hydrogen peroxide (H_2O_2) decomposition (Figure 5) into water

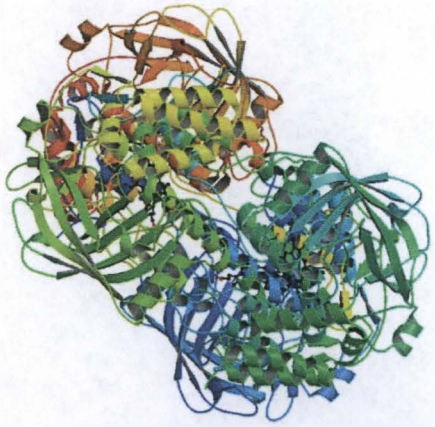

Figure 4. Crystal structure of catalase tetramer (PDB 1F4J).

and oxygen (Vlasits et al., 2007). The rate of detoxification is not dependent solely on the catalase concentration, however, and is proportional to the H_2O_2 concentration (Nicholls et al., 2000; Kirkman and Gaetani, 2006). This influence is similar to the influence of temperature on many proteins; the rate of catalase activity is lower at low concentrations of H_2O_2 and increases as the concentration increases until reaching a maximal rate of activity. If the concentration of H_2O_2 reaches high levels, catalase is inactivated and its activity rate decreases (Altomare et al., 1974; Nicholls, 2012).

Hydrogen peroxide, a reactive oxygen species (ROS), can cause extensive cellular damage, especially during periods of elevated cellular stress (Avery, 2011), and the negative effects of H_2O_2 have been hypothesized to be one of the key factors in the aging process (Harman, 1956). The Free Radical Theory of Aging suggests that aging is caused by oxidative damage from ROS over time (Harman, 1956), and that catalase overexpression in the mitochondrion has the ability to combat these effects (Schriner and Linford, 2006). Several studies have shown a high correlation between catalase activity and diabetes, and that oxidative stress might drive insulin resistance (Goth and Eaton, 2000; Henriksen, 2013), while low H_2O_2 levels have been shown to act as a cellular messenger in insulin signaling (Goth, 2008).

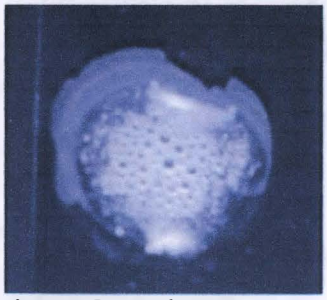

Figure 5. Hydrogen peroxide being decomposed by catalase.

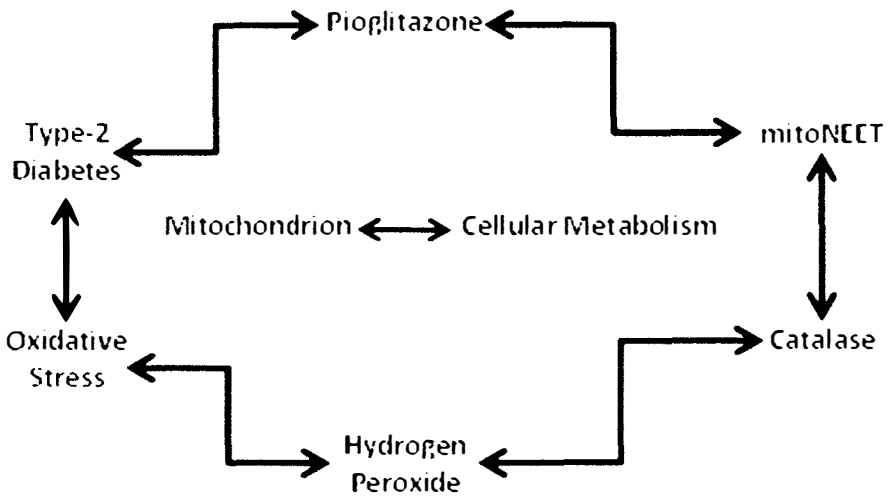

Figure 6. Flow chart representation of interactions and ties of key subjects of study.

My research focused on role of the mitochondrion in cellular metabolism and the interruption of normal cellular metabolism in type-2 diabetes, and was based upon the following known interactions: between mitoNEET and pioglitazone hydrochloride and between pioglitazone hydrochloride and type-2 diabetes (Colca et al., 2004), between oxidative stress and type-2 diabetes (Rudich et al., 1999; Maddux et al., 2001; Saengsirisuwan et al., 2001, 2004; Evans et al., 2005; Bonnard et al., 2008), and between H_2O_2 and oxidative stress and between catalase and H_2O_2 (Betteridge, 2000). By confirming a physical interaction between mitoNEET and catalase, I sought to further the understanding of the link between type-2 diabetes, mitoNEET, and catalase (Figure 6).

Because of the specific location of mitoNEET, and its interaction with anti-diabetic drugs, a possible role of the protein in diabetes treatment and/or prevention has been theorized. The purpose of my study was to explore the interactions of these two proteins in more detail. My study investigated the possible physical interaction between mitoNEET and the enzyme catalase, as well as the effect of mitoNEET on catalase activity. Based on a previous study concerning mitoNEET and another protein, I

hypothesized that mitoNEET and catalase would interact physically (M. Menze, personal communication). I also hypothesized that mitoNEET would affect the efficiency by which catalase neutralizes H_2O_2 in the cell.

Materials and Methods

Buffer Composition

The buffer solution in all of the assays used was a phosphate buffer (PBS) composed of: 3.522 g KH_2PO_4 and 7.268 g Na_2HPO_4 diluted to 1000 mL with H_2O, pH adjusted to 7.0 using hydrochloric acid. Water for solution preparation was purified with a Milli-Q Reagent Water System (Millipore, Billerica, MA) to an electrical resistance of 18 mΩ.

Concentration of Proteins

Bovine liver catalase (Sigma Aldrich, St. Louis, MO) crystals were pelleted to remove the thymol that was present as preservative. The pellet was washed with water and then pelleted again. The pellet was resuspended in PBS and stored at 4 °C. The concentration of catalase was determined using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Waltham, MA).

Soluble c-terminal mitoNEET (residue 33-108) with an added 6-HIS tag was expressed in *Escherichia coli*, and provided by Dr. Mary Konkle, Department of Chemistry, Eastern Illinois University. The purification methods to separate mitoNEET from whole bacterial lysates were adopted from previous research (Roberts, 2013). Briefly, after induction of mitoNEET production by the addition of isopropyl β-D-1-thiogalactopyranoside to the bacterial cells lysis was induced using chicken egg white lysozyme (4 mg/mL; Fisher Bioreagents, Fairlawn, NJ) and triton X-100 (0.1%, Integra, Renton, WA). The mixture was kept at 4 °C while shaken for an hour and then spun down at 108,800 g for 60 minutes. The supernatant was purified using a HisPur Ni-NTA chromatography cartridge (Fisher Scientific, Pittsburg, PA) with a fast protein liquid

chromatography system (GE® AKTApurifier, Milwaukee, WI) through a step gradient of increasing imidazole concentrations (10 mM, 20 mM, 250 mM). Because the oxidized iron-sulfur cluster appears red the colored fractions from the column eluted with 250 mM imidazole were tested for purity by SDS-PAGE (4-15% TG, BioRad Corporation, Hercules, CA). The samples consisted of 10 μL of mitoNEET and 10 μL of laemmli sample buffer loading dye with a reductant, β-mercaptoethanol (BME; Bio-Rad). Samples were incubated at 90 °C for 10 minutes and the gel was run using 10x Tris/glycine/SDS Buffer (Bio-Rad) at 200 V for approximately 30 minutes.

Samples with only minor contaminations of other bacterial proteins were pooled and dialyzed against PBS (pH = 8.5). The mitoNEET was concentrated by centrifugation to 250 μM using a 4 mL 10,000 molecular weight cut-off centrifugal filter (Merck Millipore, Billerica, MA) and the concentrated protein underwent a minimum of two freeze/thaw cycles to ensure the removal of misfolded protein. The concentrated protein was stored at -80 °C until used in the assays.

Catalase and mitoNEET Combination Spectrum

The interactions between catalase and mitoNEET were investigated utilizing an Evolution 201 UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA). A blank was taken using PBS. MitoNEET was added to the cuvette (40 μM) and a spectrum taken from λ = 244-444 nm every minute until the mitoNEET spectrum stabilized and no change was observed. A blank was taken once a change in the spectrum was not seen for three consecutive readings. Catalase was added (80 nM) to the cuvette and a spectrum taken from λ = 244-444 nm (600 nm/min, 30 cycles).

Optimization of Conditions

An absorbance curve of H_2O_2 concentration was established to identify the optimal concentration to perform the catalase assay. The standard curve was based on the absorbance of H_2O_2 at λ = 240 nm and was established by diluting a stock solution of 30% H_2O_2 to a range of final concentrations between 0.01% and 1.5%. The absorbance of hydrogen peroxide below 0.01% was unnecessary to investigate for the scope of my study. The optimal H_2O_2 concentration was chosen while within the range that follows Beer's Law and with a starting absorption value above 0.300.

The catalase concentration was optimized by performing the catalase assay (using the control solution scheme) with varying concentrations of catalase. The optimal enzyme concentration was defined as a concentration that resulted in a gradually changing hydrogen peroxide concentration over the entire 90 second period.

The incubation solution was composed of 125 μM mitoNEET and 250 nM catalase. Incubation conditions were optimized by varying the amount of time solutions were incubated at room temperature and on ice. The three time schemes tested were an hour on ice, an hour at room temperature, and 0.5 hours at room temperature followed by 0.5 hours on ice. The incubation schemes were compared based on overall H_2O_2 degradation rates by catalase and the magnitude of difference in the rates between the control solutions containing only catalase and the mixed catalase-mitoNEET solutions.

Catalase Assay

The catalase assay was performed using an Evolution 201 Spectrophotometer (Thermo Scientific) at 240 nm using 1 cm quartz cuvettes. The spectrophotometer was tared with a blank cuvette containing 860 μL PBS and 100 μL 0.6% H_2O_2. A 20 μL

sample of catalase (10 nM, pH = 7.0 in PBS) was added to 20 μL mitoNEET (250 μM, pH 8.5, in PBS) and incubated at room temperature for 30 minutes. After 30 minutes, the solution was placed on ice for 30 minutes. The 40 μL of solution was then added to the cuvette and the absorption was taken for a 90 second period with readings every 0.130 seconds. Controls were run replacing the mitoNEET sample with PBS Buffer (pH = 8.5).

Statistical Analysis

Statistical analysis comparing catalase activity rate in control and mitoNEET samples was done using student's t-test on Sigma Plot 11.0 software (Systat Software Inc., San Jose, CA).

Results

SDS-PAGE analysis demonstrates that Ni affinity is well suited to purify mitoNEET from crude bacterial lysate. All fractions analyzed that were highly pure mitoNEET were seen as a single band with a molecular weight of 12 kDa (Figure 7).

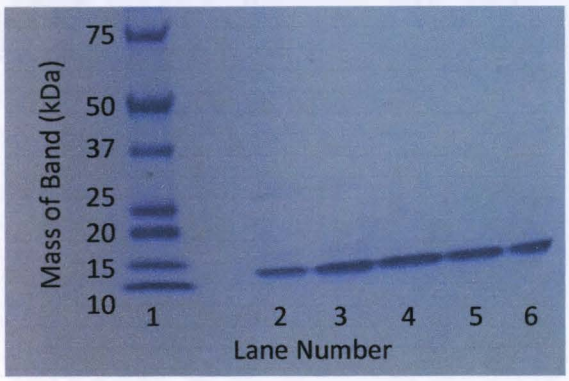

Figure 7. SDS-PAGE Gel of 12 kDa mitoNEET samples purified by nickel column chromatography (lane 2-6) and standard ladder (lane 1) stained by Coomassie blue stain.

After the purity of mitoNEET was established, any change in the spectrum of a catalase and mitoNEET mixture was investigated over a 15 minute period (Figure 8). Changes in absorption were seen in the catalase spectrum in the presence of mitoNEET over the 15 minute period. A decrease in absorption was seen at λ = 250 nm and between 300 and 380 nm. An increase in absorption was seen at λ = 270 and 400 nm and no change was seen at λ = 280 nm (Figure 8).

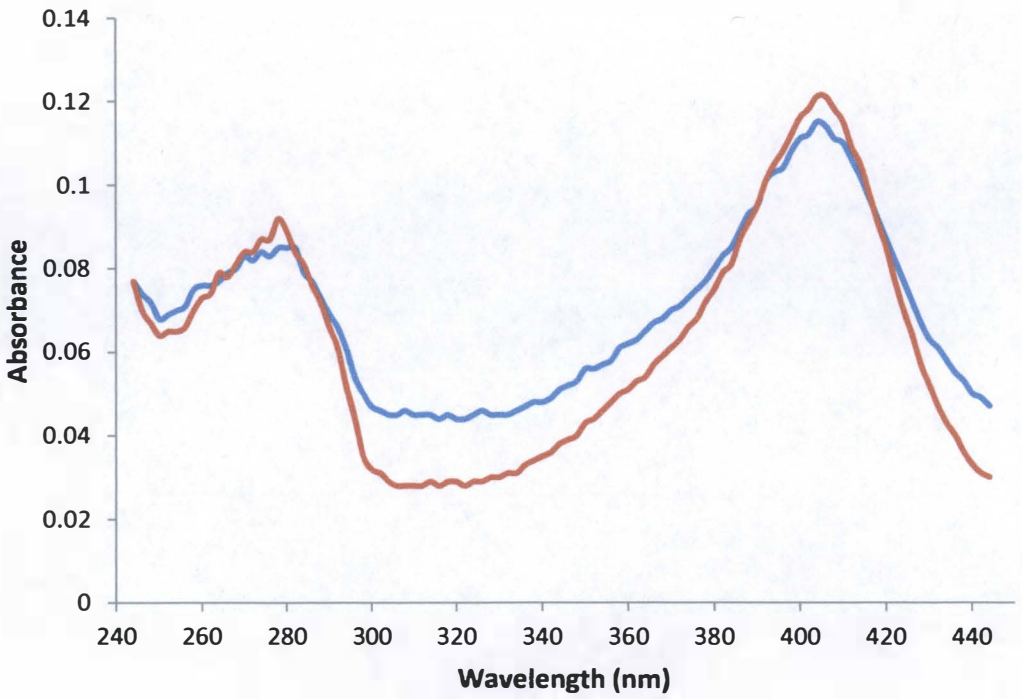

Figure 8. Initial spectrum (blue) and spectrum 15 minutes after addition (red) of a catalase and mitoNEET containing solution taken from 244-444 nm.

Hydrogen peroxide absorption and concentration share a linear relationship and follow Beer's Law between 0.01% and 0.08%. However, above a concentration of 0.08% H_2O_2, the measured absorbance showed a plateau effect (Figure 9).

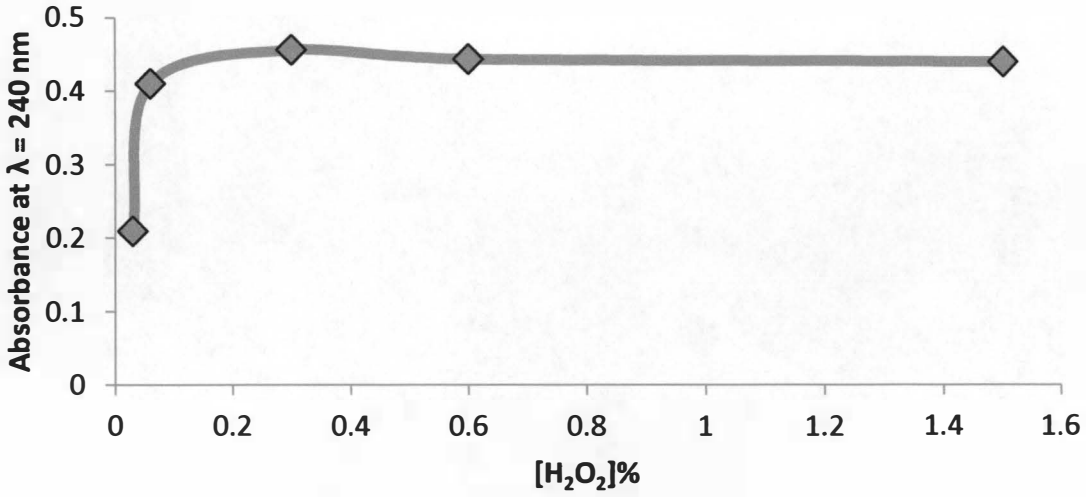

Figure 9. Concentration dependent absorbance curve of hydrogen peroxide.

The hydrogen peroxide concentration at λ = 240 nm follows Beer's Law between the concentrations of 0.01% and 0.08% with an r^2 value of 0.97. Hydrogen peroxide concentration was optimal at 0.06% when the absorbance is measured at 240 nm at pH = 7.0 (Figure 10).

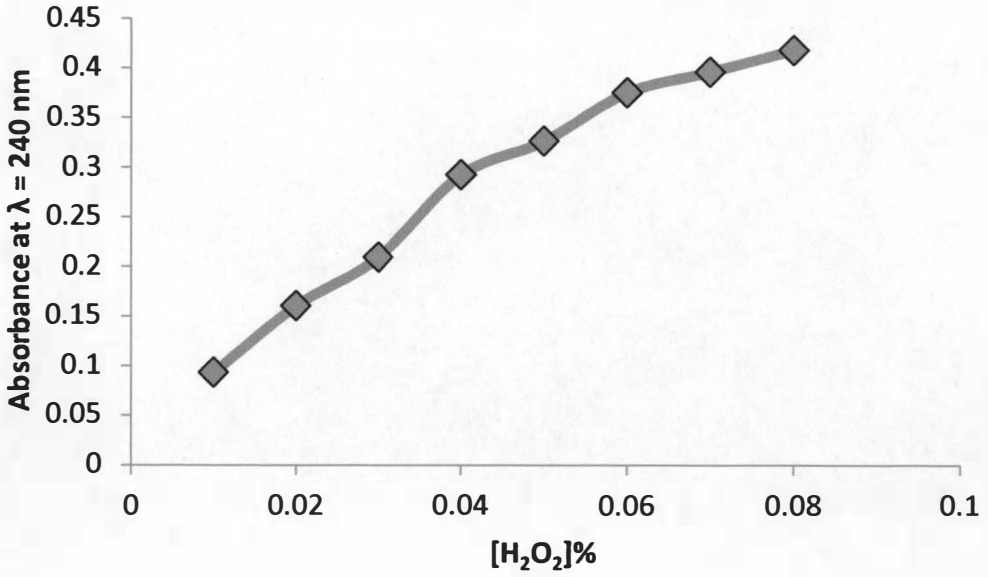

Figure 10. Concentration-dependent absorbance curve of hydrogen peroxide.

The average rate (±1 SD) of catalase activity increased after the introduction of mitoNEET. The samples containing mitoNEET had higher rates of catalase activity than the control sample in the incubation trial at room temperature for an hour and the incubation trial at room temperature for a half hour followed by a half hour on ice. Catalase activity between the mitoNEET sample was similar to the control samples in the hour on ice trial. The largest difference in the activity rate of catalase was observed between control samples (0.0858 ± 0.0103 nM H_2O_2 min^{-1} µg $protein^{-1}$; $n = 3$) and mitoNEET samples (0.1310 ± 0.0141 nM H_2O_2 min^{-1} µg $protein^{-1}$; $n = 3$), in the treatment conditions of 30 min room temperature followed by 30 min on ice (Figure 11).

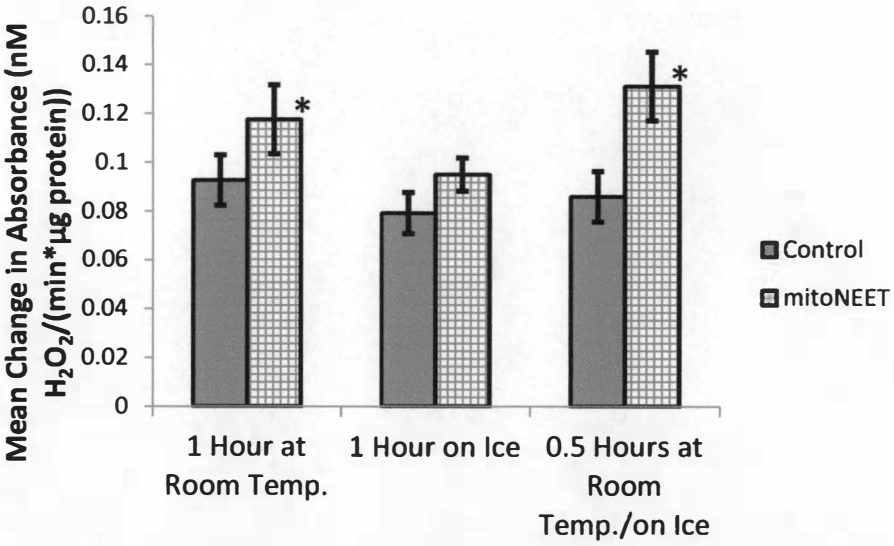

Figure 11. Mean change in catalase activity in control and mitoNEET samples at three incubation conditions ($n = 3$). * = significant difference ($p \leq 0.05$).

The average rate of catalase activity in absence of mitoNEET was 0.0899 ± 0.0195 nM H_2O_2 min^{-1} µg $protein^{-1}$ ($n = 25$), whereas the activity rate in the presence of mitoNEET was 0.1259 ± 0.0253 nM H_2O_2 min^{-1} µg $protein^{-1}$ ($n = 19$; Figure 12). The

addition of mitoNEET increased the rate of H_2O_2 decomposition by catalase by 40% (Figure 12).

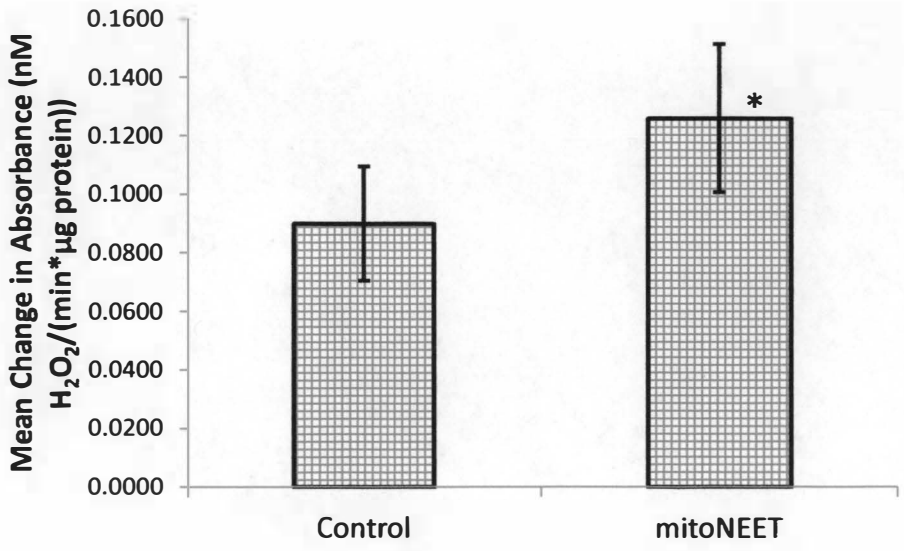

Figure 12. Mean change in absorbance of hydrogen peroxide over a 90 second period in control (n = 25) and mitoNEET (n = 15) samples. * = significant difference ($p \leq 0.05$).

Discussion

From previous studies on the interactions between mitoNEET and other proteins and compounds, I hypothesized that mitoNEET would form some type of physical interaction with catalase and affect the rate of H_2O_2 decomposition through either catalase activation or inactivation.

The hypothesis of an interaction between mitoNEET and catalase was supported. The spectrum of catalase-mitoNEET showed changes at λ = 270, 320, and 400 nm. The presence of the changes throughout the spectrum indicates that the two proteins are interacting. Unfortunately, these changes do not give any information about the specific method of interaction, leaving unclear the molecular mechanism by which the proteins interact. My findings are similar to previous studies demonstrating that mitoNEET interacts with several compounds, including reservatrol-3-sulfate, glutamate dehydrogenase 1 (GDH1), and NADPH (Arif et al., 2011; M. Menze, personal communication). Because the interaction between mitoNEET and GDH1 occurs through disulfide bonds, the possibility of disulfide bonds between catalase and mitoNEET was investigated utilizing the spectrum and SDS-PAGE results (M. Menze, personal communication). The stable reading at λ = 280 nm, and the subsequent SDS-PAGE results, does eliminate one possible method of interaction. This particular wavelength is influenced by the presence of disulfide bonds and the lack of a change at this particular wavelength is indicative that the interaction to not through disulfide bonds. The subsequent gel results (data not shown due to poor picture quality) revealed that lanes lacking (non-reducing) and containing BME (reducing) produced identical results. If mitoNEET and catalase had interacted through disulfide bonds, the lanes containing

BME would contain two bands: one band representing the mitoNEET monomer and one band representing the catalase monomer. If the two proteins interacted through a disulfide bond, the lanes without BME would have shown the same two bands, but would have also contained a third band representing the combined mass of the two proteins.

The hypothesis of an effect on catalase activity through the introduction of mitoNEET was supported. A 40% increase in H_2O_2 decomposition by catalase was seen after the introduction and incubation with mitoNEET. Although catalase is an efficient enzyme, and has one of the highest k_{cat} values of enzymes, it is not diffusion-limited (Rousse, 2012).

$$\text{Catalase } Fe^{3+} + H_2O_2 \rightarrow \text{Catalase } FeO^{3+} + H_2O$$
$$\text{Catalase } FeO^{3+} + H_2O_2 \rightarrow \text{Catalase } FeO^{3+} + O_2 + H_2O$$
$$\text{Catalase } FeO^{3+} + AH \rightarrow \text{Catalase } FeOH^{3+} + A$$
$$\text{Catalase } FeOH^{3+} + AH \rightarrow \text{Catalase } Fe^{3+} + A + H_2O$$

Figure 13. Mechanism of hydrogen peroxide decomposition by catalase

Catalase utilizes an iron molecule in the breakdown of H_2O_2 and is in the Fe^{3+} state at rest (Figure 13; Nicholls, 2012). Because mitoNEET increases the rate of catalase activity, and because catalase is not diffusion-limited (Roussel, 2012), it is possible that mitoNEET is helping one of the rate limited transition steps of the H_2O_2 decomposition. Because both proteins (mitoNEET and catalase) contain an iron molecule, and because the iron in catalase is directly involved in H_2O_2 decomposition, there are several possible avenues for this interaction. One possibility is that the iron in the mitoNEET heme reduces the catalase iron, returning it to the rest state.

The increase in the catalase activity rate is consistent with data collected from the spectrums of catalase-mitoNEET in supporting an interaction between catalase and mitoNEET, yet neither indicate the exact method of interaction. Similar effects as the

effects on catalase by mitoNEET were seen with mitoNEET and GDH (M. Menze, personal communication).

My research has validated an interaction between mitoNEET and catalase that is involved with cellular energy production and oxidative stress. There are multiple directions for the project to pursue in the future. One direction will be to further investigate the physical interaction between mitoNEET and catalase. Because the exact method of the physical interaction was not determined, this will be necessary to understand the effects of that interaction. Other future directions include investigating the effects of adding pioglitazone hydrochloride to the incubation, as well as the effect of varying concentrations of mitoNEET or overexpression *in vivo*. The mechanism and possible roles of mitoNEET are still poorly understood and much research is still needed to explore the possibilities of this protein.

Literature Cited

Altomare, R.E., Kohler, J., Greenfield, F. and Kittrell, J.R. (1974). Deactivation of immobilized beef liver catalase by hydrogen peroxide. *Biotechnology and Bioengineering* 16: 1659-1673.

Anderson, E.J., Lustig, M.E., Boyle, K.E., Woodlief, T.L., Kane, D.A., Lin, C., Price, J.W., Kang, L., Rabinovitch, P.S., Szeto, H.H., Houmard, J.A., Cortright, R.N., Wasserman, D.H. and Neufer, P.D. (2009). Mitochondrial H_2O_2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *Journal of Clinical Investigation* 119: 573-581.

Arif, W., Xu, S., Isailovic, D., Geldenhuys, W.J., Carroll, R.T. and Funk, M.O. (2011). Complexes of the outer mitochondrial membrane protein mitoNEET with resveratrol-3-sulfate. *Biochemistry* 50: 5806-5811.

Avery, S.V. (2011). Molecular targets of oxidative stress. *Biochemical Journal* 434: 201-210.

Bak, D.W. and Elliott, S.J. (2013). Conserved hydrogen bonding networks of mitoNEET tune Fe-S cluster binding and structural stability. *Biochemistry* 52: 4687-4696.

Baxter, E.L., Zuris, J.A., Wang, C., Vo, P.L.T., Axelrod, H.L., Cohen, A.E., Paddock, M.L., Nechushtai, R., Onuchic, J.N. and Jennings, P.A. (2012). Allosteric control in a metalloprotein dramatically alters function. *Proceedings of the National Academy of Sciences* 110: 948-953.

Betteridge, D.J. (2000). What is oxidative stress? *Metabolism* 49: 3-8.

Bonnard, C., Durand, A., Peyrol, S., Chariseaume, E., Chauvin, M.A., Morio, B., Vidal, H. and Rieusset, J. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *Journal of Clinical Investigation* 118: 789-800.

Center for Disease Control and Prevention (2014, April 17). *Diabetes Public Health Resource*. Retrieved from http://www.cdc.gov/Diabetes/

Chang, N.C., Nguyen, M., Germain, M. and Shore, G.C. (2009). Antagonism of beclin 1-dependent autophagy by BCL-2 at the endoplasmic reticulum requires NAF-1. *EMBO Journal* 29: 606-618.

Chen, Y.F., Wu, C.Y., Kirby, R., Kao, C.H. and Tsai, T.F. (2010). A role for the CISD2 gene in lifespan control and human disease. *Annals of the New York Academy of Sciences* 1201: 58-64.

Colca, J.R., McDonald, W.G., Waldon, D.J., Leone, J.W., Lull, J.M., Bannow, C.A., Lund, E.T. and Methews, W.R. (2004). Identification of a novel mitochondrial protein ("mitoNEET") cross-linked specifically by a thiazolidinedione photoprobe. *American Journal of Physiology* 286: 252-260.

Conlan, A.R., Axelrod, H.L., Cohen, A.E., Abresch, E.C., Zuris, J., Yee, D., Nechustai, R., Jennings, P.A. and Paddock, M.L. (2009a). Crystal structure of miner1: The redox-active 2Fe-2S protein causative in Wolfram syndrome 2. *Journal of Molecular Biology* 392: 143-153.

Conlan, A.R., Paddock, M.L., Axelrod, H.L., Cohen, A.E., Abresch, E.C., Wiley, S., Roy, M., Nechushtai, R. and Jennings, P.A. (2009b). The novel 2Fe-2S

outer mitochondrial protein mitoNEET displays conformational flexibility in its N-terminal cytoplasmic tethering domain. *Acta Crystallographica Section F Structural Biology Communications* 65: 654-659.

Dicus, M.M., Conlan, A., Nechushtai, R., Jennings, P.A., Paddock, M.L., Britt, R.D. and Stoll, S. (2010). Binding of histidine in the $(Cys)_3(His)_1$-coordinated [2Fe-2S] cluster of human mitoNEET. *Journal of American Chemistry Society* 132: 2037-2049.

Evans, J.L., Maddux, B.A. and Goldfine, I.D. (2005). The molecular basis for oxidative stress-induced insulin resistance. *Antioxidants and Redox Signaling* 7: 1040-1052.

Geldenhuys, W.J., Funk, M.O., Awale, P.S., Lin, L. and Carroll, R.T. (2011). A novel binding assay identifies high affinity ligands to the rosiglitazone binding site of mitoNEET. *Bioorganic & Medicinal Chemistry Letters* 21: 5498-5501.

Goth, L. (2008). Catalase deficiency and type-2 diabetes. *Diabetes Care* 31: 93.

Goth, L. and Eaton, J. (2000). Hereditary catalase deficiencies and increased risk of diabetes. *Lancet* 356: 1820-1821.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology* 2: 298-300.

Henriksen, E.J. (2013). Effects of H2O2 on insulin signaling the glucose transport system in mammalian skeletal muscle. *Methods in Enzymology* 528: 269-278.

Jitrapakdee, S., Wutthisathapornchai, A., Wallace, J.C. and MacDonald, M.J. (2010). Regulation of insulin secretion: role of mitochondrial signaling.

Diabetologia 53: 1019-1032.

Kirkman, H.N. and Gaetani, G.F. (2006). Mammalian catalase: a venerable enzyme with new mysteries. *Biochemical Sciences* 32: 44-50.

Kusminski, C.M., Holland, W.L., Sun, K., Park, J., Sprugin, S.B., Lin, Y., Askew, G.R., Simcox, J.A., McClain, D.A., Li, C. and Scherer, P.E. (2012). MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. *Nature Medicine* 18: 1539-1549.

Landry, A.P. and Ding, H. (2014). Redox control of human mitochondrial outer membrane protein mitoNEET [2Fe-2S] clusters by biological thiols and hydrogen peroxide. *Journal of Biological Chemistry* 289: 4307-4315.

Lin, J., Zhou, T., Ye, K. and Wang, J. (2007). Crystal structure of human mitoNEET reveals distinct groups of iron-sulfur proteins. *Proceedings of the National Academy of Sciences* 104: 14640-14645.

Loew, O. (1900). A new enzyme of general occurrence in organisms. A preliminary note. *Science* 11: 701-702.

Maddux, B.A., See, W., Lawrence, J.C., Goldfine, A.L., Goldfine, I.D. and Evans, J.L. (2001). Protection against oxidative stress-induced insulin resistance in rat L6 muscle cells by micromolar concentrations of α-lipoic acid. *Diabetes* 50: 404-410.

Maiuri, M.C., Criollo, A. and Kroemer, G. (2010). Crosstalk between apoptosis and autophagy within the Beclin 1 interactome. *EMBO Journal* 29: 515-516.

Napier, I., Ponka, P. and Richardson, D.R. (2005). Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood* 105: 1867-1874.

National Diabetes Education Program (2011). *The Facts About Diabetes: A Leading Cause of Death in the U.S.* Retrieved from http://ndep.nih.gov/diabetes-facts/index.aspx

Nechushtai, R., Conlan, A.R., Harir, Y., Song, L., Yogev, O., Eisenberg-Domovich, Y., Michaeli, D., Rosen, R., Ma, V., Luo, Y., Zuris, J.A., Paddock, M.L., Cabantchik, Z.I., Jennings, P.A. and Mittler, R. (2012). Characterization of *Arabidopsis* NEET reveals an ancient role for NEET proteins in iron metabolism. *Plant Cell* 24: 2139-2154.

Nicholls, P. (2012). Classical catalase: Ancient and modern. *Archives of Biochemistry and Biophysics* 525: 95-101.

Nicholls, P., Fita, I. and Loewen, P.C. (2000). Enzymology and structure of catalases. *Advances in Inorganic Chemistry* 51: 51-106.

Paddock, M.L., Wiley, S.E., Axelrod, H.L., Cohen, A.E., Roy, M., Abresch, E.C., Capraro, D., Murphy, A.N., Nechustai, R., Dixon, J.E. and Jennings, P.A. (2007). MitoNEET is a uniquely folded 2Fe-2S outer mitochondrial membrane protein stabilized by pioglitazone. *Proceedings of the National Academy of Sciences* 104: 14342-14347.

Patti, M.E. and Covera, S. (2010). The role of mitochondria in the pathogenesis of type-2 diabetes. *Endocrine Review* 31: 364-395.

Ramadori, G., Gautron, L., Fujikawa, T., Vianna, C.R., Elmquist, J.K. and Coppari, R. (2009). Central administration of resveratrol improves diet-induced diabetes. *Endocrinology* 150: 5326-5333.

Rigoli, L. and Di Bella, C. (2012). Wolfram syndrome 1 and Wolfram syndrome 2.

Current Opinion in Pediatrics 24: 512-517.

Roberts, M.E. (2013). *Characterization of the Stability and Reactivity of MitoNEET: A Novel Diabetes Drug Target* (Master's thesis). Eastern Illinois University, Charleston, IL.

Roussel, M.R. (2012). *A Life Scientist's Guide to Physical Chemistry*. Cambridge: Cambridge University Press.

Rudich, A., Tirosh, A., Potashnik, R., Khamaisi, M. and Bashan, N. (1999). Lipoic acid protects against oxidative stress induced impairment in insulin stimulation of protein kinase B and glucose transport in 3T3-L1 adipocytes. *Diabetologia* 42: 949-957.

Saengsirisuwan, V., Kinnick, T.R., Schmit, M.B. and Henriksen, E.J. (2001). Interactions of exercise training and lipoic acid on skeletal muscle glucose transport in obese Zucker rats. *Journal of Applied Physiology* 91: 145-153.

Saengsirisuwan, V., Perez, F.R., Sloniger, J.A., Maier, T. and Henriksen, E.J. (2004). Interactions of exercise training and alpha-lipoic acid on insulin signaling in skeletal muscle of obese Zucker rats. *American Journal of Physiology – Endocrinology and Metabolism* 287: 529-536.

Schriner, S.E. and Linford, N.J. (2006). Extension of mouse lifespan by overexpression of catalase. *Age* 28: 209.

Schriner, S.E., Linford, N.J., Martin, G.M., Treuting, P., Ogburn, C.E., Emond, M., Coskun, P.E., Ladiges, W., Wolf, N., Van Remmen, H., Wallace, D.C. and Rabinovitch, P.S. (2005). Extension of murine life span by overexpression of catalase targeted mitochondria. *Science* 308: 1909-1911.

Supale, S., Li, N., Brun, T. and Maechler, P. (2012). Mitochondrial dysfunction in pancreatic B cells. *Trends in Endocrinology and Metabolism* 23: 477-87.

Tsai, T.F., Chen, Y.F. and Tsai, S.F. (2009). CISD2-knockout mice and uses thereof. U.S. Patent Appl 12/481.

Vlasits, J., Jakopitsch, C., Schwanninger, M., Holubar, P. and Obinger, C. (2007). Hydrogen peroxide oxidation by catalase-peroxidase follows a non-scrambling mechanism. *FEBS Letters* 581: 320-324.

Wiederkehr, A. and Wollheim, C.B. (2006). Minireview: implication of mitochondria in insulin secretion and action. *Endocrinology* 147: 2643-2649.

Wiley, S.E., Murphy, A.N., Ross, S.A., van der Geer, P. and Dixon, J.E. (2006). MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity. *Proceedings of the National Academy of Sciences* 104: 5318-5323.

Wiley, S.E., Paddock, M.L., Abresch, E.C., Gross, L., van der Geer, P., Nechushtai, R., Murphy, A.N., Jennings, P.A. and Dixon, J.E. (2007). The outer mitochondrial membrane protein mitoNEET contains a novel redox-active 2Fe-2S cluster. *Journal of Biological Chemistry* 282: 23745-23749.

Zamocky, M. and Koller, F. (1999). Understanding the structure and function of catalase: clues from molecular evolution and *in vitro* mutagenesis. *Progress in Biophysics and Molecular Biology* 72: 19-66.

Zhou, T., Lin, J., Feng, Y. and Wang, J. (2010). Binding of reduced nicotinamide adenine dinucleotide phosphate destabilizes the iron-sulfur clusters of

human mitoNEET. *Biochemistry* 49: 9604-9612.

Zuris, J.A., Harir, Y., Conlan, A.R., Shvartsman, M., Michaeli, D., Tamir, S., Paddock, M.L., Onuchic, J.N., Mittler, R., Cabantchik, Z.I., Jennings, P.A. and Nechustai, R. (2011). Facile transfer of [2Fe-2S] clusters from the diabetes drug target mitoNEET to an apo-acceptor protein. *Proceedings of the National Academy of Sciences* 108: 13047−13052.