UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effects of T1-CCDC90B on the Mitochondria in Human Mammary Epithelial Cells

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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2017
Dedication

I would like to thank Dr. Jean Wang for being my PI at UCSD, for putting up with me and most importantly helping guide and teach me through this long process. I would also like to thank Dr. Richard Hampton and Dr. Amy Kiger for taking the time to be on my committee and for being amazing professors who I was lucky to take and work with.

I would also like to thank all the members of Dr. Jean Wang’s lab for the moral support and guidance whenever necessary. I would like to thank Daniel Quan for his mentorship. Lastly, I would like to thank my family and friends for supporting me.
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ABSTRACT OF THE THESIS

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Coiled-Coil Domain Containing 90B (CCDC90B) is a protein of unknown function, which has three functional transcripts according to Ensembl genome browser. In this project, we showed that T1-CCDC90B does, in fact, localize to the mitochondria in MCF-10A cells as there was co-localization via immunofluorescence between CCDC90B protein staining and the activity of the mitochondria. We also showed that when we overproduced T1-CCDC90B protein, there was an increase in COX1 protein in MCF10A cells. An increase in the T1 transcript also caused an increased in the mitochondrial potential per total mitochondrial compartment size and this difference was
directly due to a reduction in the size of the mitochondrial compartment. Furthermore, we reversed these effects by stable knockdown of CCDC90B protein in cells and saw an increase in the mitochondrial compartment size.
I. Introduction
1.1 The mitochondrion and mitochondrial structure

All life on earth requires energy to survive and perform a necessary set of functions. The mitochondria are a vital organelle found in the cytosol of eukaryotic cells and has many functions, but the primary function is to create energy for the cell. Due to its important role, there are typically multiple mitochondrion found in the cell's cytosol, but the concentration of the organelle can vary depending on the type of cell and its function.

The mitochondrion has optimized its structure to maximize its ability to perform its unique functions for the cell. The mitochondrion is a double membrane-bound organelle and both the outer and inner membrane are composed of phospholipid bilayers (Kuhlbrandt, 2015). The outer membrane contains multiple transmembrane proteins and pores, which allow the outer membrane to be freely permeable to smaller molecules such as ions, nutrient molecules, ATP, etc, and thus can diffuse into the intermembrane space unimpeded. The inner membrane, however, is selectively permeable and only allows oxygen, carbon dioxide, and water to diffuse without assistance. The inner membrane is complex and the primary site of ATP (energy) production and synthesis, therefore, many proteins and protein complexes necessary to the energy production process are embedded in the membrane (Kuhlbrandt, 2015). The protein complexes embedded in the inner membrane include the four complexes in the electron transport chain (ETC and the ATP synthase protein complex all five of which are composed of multiple protein subunits and each complex plays a direct role in aerobic respiration and the production of ATP. Due to the crucial role of the inner membrane proteins, the inner membrane can fold into organized layers known as cristae. These folds increase the surface area and allow for an
increase in the concentration of proteins and protein complexes located in the mitochondrial inner membrane. (Kuhlbrandt, 2015). Finally, the last major structure of the mitochondria is the mitochondrial matrix. The mitochondrial matrix is a gel-like fluid that fills the space within the inner membrane. The matrix has a high concentration of protein and enzymes to help carry out the mitochondria’s functions. The mitochondrial matrix houses both the ribosomes and the mitochondrial genomic content in the form of DNA.

The mitochondrion has many cellular functions. The primary function of the mitochondria is metabolism, specifically the production of energy in the form of ATP. This process occurs through a mechanism known as aerobic respiration, which is oxygen dependent (Rich, 2003). Aerobic respiration must go through three main steps to produce ATP: the glycolysis step, the TCA cycle, and the electron transport chain (ETC). ATP is ultimately synthesized through the ATP synthase protein complex located in the inner membrane of the mitochondria. ATP is made by using the energy stored in the proton gradient, which is created by pumping protons into the intermembrane space by the four protein complexes in the ETC (Rich, 2003). The protons are able to be pumped from the matrix to the intermembrane space against its concentration gradient by using the energy released from redox reactions through electron donor molecules, which are created during the glycolysis and the TCA cycle processes (see 1.3 and 1.4). The proton gradient created by the ETC, also known as membrane potential, is one measure used to detect how active and healthy the mitochondria of a cell is which is important for the cell’s health and function (Duchen, 2004). In addition to energy production, the mitochondriion have an expanded role in metabolism as it is involved in the catabolism, anabolism, and recycling
of certain biochemical which is imperative for the proper health and function of the cell. The mitochondria, however, doesn't just play a role in metabolism as the organelle is required for many other cellular functions. One such function is the role it plays in programmed cell death or apoptosis (Youle, 2009). Apoptosis is a process in which biochemical reactions occur to cause changes in the cell which will eventually lead to the death of the cell. Apoptosis plays an important role in embryo development, but also removes abnormal cells that can hurt the organism such as those that are damaged (such as cancerous cells) or infected. The role of the mitochondria in apoptosis is to facilitate apoptosis as a proapoptotic protein and insert itself into the mitochondrial membrane to forms pores in the membrane allowing the cytochrome c proteins to be released into the cytosol, which causes an activation cascade of caspase proteins eventually leading to apoptotic death of the cell. An additional role of the mitochondria is the storage of calcium (Ca) ions (Nicholls, 2004). Calcium plays a key role in biochemistry as it is involved in signal transduction, contraction of muscles, the release of neurotransmitters, enzyme function (a coenzyme), etc. In many mammals, the mitochondria in the bone tissue are the primary site of mineral storage; however, the mitochondria in other cells still have a minor role in Ca storage. Finally, the mitochondria have other specific roles which can be dependent on cell type. As mentioned above the mitochondria plays a key role in metabolism but depending on cell type it can be specialized to metabolize cholesterol, neurotransmitters or ammonia (detoxification). The mitochondrion can also contribute to the synthesis of certain hormones such testosterone and estrogen (Rosier, 2006).
1.2 Endosymbiotic Theory and the Mitochondrial Genome

Despite the role mitochondria play presently among many eukaryotic organisms, the species responsive for one of the most vital components of the cell resemble the proteobacteria known as *Rickettsia* (Andersson, 1998). It was once suggested that the mitochondria was a separate organism that had a similar role to the organelle in our cells today, but at one point was incorporated into the eukaryotic cells and co-evolved together. This theory was first proposed by Lynn Margulis in the late 1960s and is known as the endosymbiotic theory, however, was only given recognition later. One of the first observations about the mitochondria that supported this theory is that it has its own double bilayer like prokaryotes. It is also known that the mitochondria has its own circular genome, which lacks introns; and that the central dogma of the mitochondria, the transcription and translation process occurs in succession - both of which are characteristics seen in prokaryotes (Taanman, 1999). In addition, it was shown that the mitochondria can replicate by binary fission, which is a process typically seen in bacteria (Kiefel, 2004). Based on this evidence, it is now accepted that the mitochondria at one point were a separate organism (likely a prokaryote) and were incorporated into the eukaryotic cell and both co-evolved to form what we consider the eukaryotic cell we see today.

The mitochondrial genome has been sequenced by Anderson et al. in 1981. Unlike most genetic material which is found in the nucleus and packaged in chromosomes, the mitochondrial DNA is circular and double-stranded located in the mitochondrial matrix. The mitochondrial DNA consists of 16,569 b.p. which encodes
for 37 genes which include 2 rRNAs, 22 tRNAs, and 13 mitochondrial proteins. All of
the proteins encoded in the mitochondrial genome stay in the mitochondria and play a
role in the aerobic respiration process. In fact, all the polypeptides are sent to the inner
membrane and participate as subunits to the proton complexes along the ETC.
Although the mitochondria contain its own genome, most of the mitochondria
components are imported from the nuclear genome. Based on proteomic analyses it is
known that less than 1% of the total proteins that reside in the mitochondria are from
the mitochondrial genome, while the rest are imported from the nuclear genome
(Wiedermann, 2004). Studies have also shown that most higher organisms including
mammals do not import tRNA although it is known that rRNA does get imported in
addition to proteins (Entelis, 2001).

1.3 Mitochondria and Aerobic Respiration

Nearly all evolved life forms require oxygen to function as it plays a key role in
the production of energy through aerobic respiration (Cheema, 2011). Aerobic
respiration is the process which causes the release of energy through the catabolism of
certain organic molecules in the presence of oxygen (Rich, 2003). The process starts as
carbohydrates are broken down into glucose during digestion via enzymes which gets
transported into the cell through glucose transporters (Zhao, 2007). After glucose
enters certain cells, it gets broken down in the cytosol in a process known as
glycolysis. This step is anaerobic and at the end of glycolysis 2 net ATP and 2 NADH
molecules are produced and each glucose molecule is oxidized into 2 pyruvate
molecules. Each pyruvate molecule can enter the mitochondria through an unknown transporter and in the matrix, it is oxidized into Acetyl Coenzyme A via pyruvate dehydrogenase forming an additional NADH molecule. After each Acetyl Coenzyme A molecule enters the TCA cycle, which is also located in the matrix, various enzymatic reactions occur and creates 1 ATP, 3 NADH, and a FADH$_2$ molecules.

After the TCA cycle is complete, each glucose molecule creates 10 NADH, 2 FADH$_2$, and 4 ATP molecules. Up to this stage in aerobic respiration just 4 ATP molecules are produced, the bulk of the ATP is produced during the ETC step of aerobic respiration (1.4) by using electron donors such as NADH and FADH$_2$. The NADH and FADH$_2$ molecules donate electrons into ETC and through each redox reaction along the ETC free energy is released, which is used to pump protons into the intermembrane space from the matrix against its electrochemical gradient. In order to restore the electrochemical gradient, protons flow through ATP synthase complex and this catalyzes the phosphorylation of ADP to create ATP molecules (more detailed information on this process in 1.4). Through the entire aerobic respiration process, it is estimated that each glucose molecule creates 36 net ATP molecules 32 of which come from ETC step. The aerobic respiration process can also occur through the catabolism of fats or certain proteins and enter as intermediates of the TCA cycle, therefore, it is not carbohydrate dependent process.
1.4 Electron Transport Chain (ETC) and membrane potential

As mentioned earlier, the electron transport chain (ETC) is the final step in the aerobic respiration process and is the only step that directly uses oxygen. The ETC consists of four protein complexes embedded in the inner membrane of the mitochondria. The NADH and FADH$_2$ molecules formed from the processes prior to ETC are used in the ETC to create a proton gradient through energy released in a series of redox reactions. The first protein complex in this step is NADH dehydrogenase, (Complex I) it oxidizes NADH back to NAD$^+$ which then transfers the electrons to the molecule coenzyme Q. The process of transferring electrons via redox reaction from NADH to complex I and from complex I to coenzyme Q each release enough free energy to allow protons to be pumped from the mitochondrial matrix to the intermembrane space - against its electrochemical gradient. Complex II, or succinate dehydrogenase, is the second protein complex on the ETC. Where NADH is oxidized through complex I, the FADH$_2$ is oxidized in complex II. Like NADH, FADH$_2$ gets oxidized by complex II and the electrons also get transferred to coenzyme Q; however, unlike with complex I the standard free energy released isn’t great enough to pump protons thus the reason FADH$_2$ produces less ATP than NADH. The next protein complex is known as coenzyme Q-cytochrome $c$ reductase (complex III) and acts as an intermediate for the electrons being passed from coenzyme Q to cytochrome C (Cyt $c$). As the electrons are being passed both to complex III and from complex III to Cyt $c$ protons are pumped into the intermembrane space. The final protein complex is known as cytochrome $c$ oxidase (complex IV) as it accepts the electrons from Cyt $c$ and transfers them to oxygen, the final electron acceptor, to form water. Similar to the other redox reactions along the ETC, this transfer of
electrons also causes protons to be pumped from the matrix to the intermembrane space. The electrochemical gradient builds as the protons are pumped against its concentration gradient which is needed to the synthesis of ATP. Once the electrochemical gradient is strong enough, due to the high proton concentration the gradient is restored as protons can go down its gradient through a protein complex known as F₁F₀ ATP synthase and re-enter the mitochondrial matrix. The passing of protons through the ATP synthase protein catalyzes the production of ATP via phosphorylation of ADP.

The electrochemical gradient created by pumping protons is also known as the mitochondrial membrane potential. The mitochondrial membrane potential is important to the health of the cell and changes in the potential can alter the physiological role of the cell. For example, significant changes in the mitochondrial potential (typically due to extreme stress) such as a significant decrease will reduce ATP substantially. Conversely, a significant increase in mitochondrial potential will damage the cell by ROS. Both extremes can damage the cell and in some cases, will lead to apoptosis. Where larger changes in the potential can be fatal, smaller mitochondrial potential changes tend to signal the cell either directly or indirectly for a specific function. An example of this is that a smaller change in potential can alter both the general ion flow in and out of the mitochondria, therefore affecting the mitochondrial volume (1.6) and specific ion concentrations which can cause multiple secondary effects via cell signaling. These smaller changes in potential, for example, can signal the cell to proliferate or go through apoptosis depending on the circumstance. The mitochondrial potential is critical to cells health and has a huge physiological effect on the cell; deviations in the potential can effects the cell by altering its physiological role.
1.5 Cytochrome c oxidase assembly

Cytochrome c oxidase (COX or complex IV) is the last protein complex along the ETC. Like the other 3 complexes along ETC, COX primary function is to both pump protons into the intermembrane space and transport electrons to next electron acceptor. In the COX complex, three of the subunits (COX1, COX2, and COX3) are from the mitochondrial genome and they assemble with the imported nuclear-encoded proteins to make the 14-protein subunit complex (Dennerlein & Rehling, 2015). Although the complex contains 14 subunits, it takes many more proteins and other factors to stabilize and protect the intermediates of the complex (Dennerlein & Rehling, 2015). The imported subunits from the nuclear-genome enter the mitochondria and integrate with mitochondrially encoded subunits to form a COX intermediate, MITRAC. A number of proteins associated with MITRAC constantly change as new components and cofactors are sequentially added and removed to help with COX assembly process and maturation (Dennerlein & Rehling, 2015). Throughout the process of COX assembly, MITRAC interacts with TIM to incorporate nuclear-encoded subunits into the complex, but is unknown at which stage in the process the assembly occurs. Little is known about the COX assembly process; however, it appears that COX1, a mitochondrially-encoded subunit in the COX complex plays a significant role in the process. The COX1 subunit may be the first protein in the COX assembly process and it interacts not just with other subunits, but other proteins to help mature, stabilize, and assemble the COX complex (Dennerlien, 2015). In addition to helping with the assembly process, heme is integrated
into the COX1 subunit to aid with the redox reactions (Fontanesi, 2013). By studying patients with COX deficiency, it was noted that multiple separate mutations that lead to COX deficiency the COX1 subunit levels were either decreased or not stable thus suggesting a role in COX assembly (Dennerlein & Rehling, 2015).

1.6 The Proton Gradient Can Also Regulate the Mitochondrial Matrix Volume

The homeostasis of mitochondrial volume is closely regulated as the proper volume is key to the integrity and structure of the mitochondria. Like mitochondrial membrane potential, changes in mitochondrial volume can affect the physiological role of the mitochondria. Protons play an important role in mitochondrial volume regulation as protons can enter the mitochondrial matrix not just through the ATP synthase protein, but through ion channels as well. The mitochondrial matrix volume is heavily impacted by the osmotic balance between the cytosol and mitochondria, thus the flow of ions will impact the flow of water in and out of the mitochondria. The three ions that play the biggest role in the regulation of mitochondrial matrix volume are calcium (Ca), potassium (K), and sodium (Na) in which K has the highest concentration. Because the inner membrane is impervious to ions, the concentration of these molecules is dependent on transporters and specific channels. The potassium influx is regulated by both ATP and calcium-dependent channels while its efflux is dependent on a K⁺/H⁺ antiporter. The influx of sodium ions is dependent on Na⁺/Ca²⁺ antiporter while the efflux occurs through a Na⁺/H⁺ antiporters. Finally, calcium influx is regulated by a calcium channel where the efflux of calcium is done through the Na⁺/Ca²⁺ antiporter. So, the protons can restore its
electrochemical gradient not just by making ATP, but through ion channels as well which directly affect the mitochondrial volume. Any change in the flux of these ions will alter the osmotic balance, which affects the mitochondrial volume and similar to the mitochondrial membrane potential alters the physiological role of the mitochondria. For example, extreme swelling of the mitochondria is typically seen in unhealthy and inactive mitochondria and will eventually lead to apoptosis of the cell (Gao et al., 2011). This extreme swelling damages the mitochondria as it compromises the integrity of the outer membrane, which can be fatal to the health of the mitochondria thus the cell. Although extreme swelling can be fatal, previous studies have shown that up to a 2 to 3 fold increase in the mitochondrial volume will not compromise the integrity of the outer membrane thus the mitochondria could still be healthy and active. The homeostasis of the mitochondrial volume may also play a role in ROS formation (Ferranti et al., 2002) as a correlation of ROS was seen with a hyperactive potassium channel. The volume can also play a role in cell signaling such as the uncoupling of the proton gradient (Holmuhamedov et al., 2004) and the oxidation of fatty acids (Halestrap et al., 1987).
1.7 CCDC90B protein

The protein CCDC90B (coiled-coil domain containing 90B) is a polypeptide with an unknown function that has three functional isoforms. The three isoforms are T1 (245 amino acids), T3 (254 amino acids), and the T6 (153 amino acids) isoforms (figure 1). So far our lab has been focused on the T1 transcript of CCDC90B gene which is located on human chromosome 11. As stated previously, the function of T1-CCDC90B is unknown; however, it is known that the protein coding sequence has a mitochondrial targeting sequence and a transmembrane helix (figure 1). Previous proteolytic analysis has shown that the protein is believed to be in the mitochondria with its C terminus in the inter membrane space as the transmembrane helix is embedded in the inner membrane (Gralle, Schäfer et al. 2010). Most of the coding sequence (76%) contains an evolutionarily conserved amino acid sequence found in all eukaryotic cells - DUF1640 (domain of unknown function 1640) which contains the coiled-coil domain sequence of the protein (figure 1).

A previous study showed that CCDC90B protein was also one of the genes isolated in a genome-wide CRISPR death screen of K562 CML cells which revealed proteins required for oxidative phosphorylation (Arroyo et al,2016). They performed the experiment by screening for cells which died under galactose conditions but survived in glucose growth conditions; however, this data has not been validated. A paralog of CCDC90B known as CCDC90A was originally thought to be involved as a component of the mitochondrial calcium uniporter (Vais et al.2012); however, this was later disputed and has been suggested to regulate Complex IV (COX) of the
electron transport chain (Paupe et al. 2015). It is also known that immunoprecipitation of CCDC90B resulted in a “strong” co-immunoprecipitation with CCDC90A (Tomar, et al, 2016), a finding that we also validated in our lab. Previous studies have shown that in yeast, the ortholog of the protein, known as Fmp32, is also a mitochondrial protein and the ∆fmp32 strain of yeast has the phenotype of slow growth on glycerol but not on glucose. It is currently unknown if the human CCDC90B protein is also involved in the regulation of COX. However, our lab has data showing that the CCDC90B protein is not required for human cell growth in glucose-free conditions.
II. Results and Discussion
For my thesis project, I was tasked with investigating both the location and the function of T1-CCDC90B protein in MCF-10A cells. MCF-10A cells are adherent non-tumorigenic breast epithelial cells. To find the location and the function of T1-CCDC90B, we created MCF-10A cell lines for both the overexpression of the T1 isoform and knockdown of the CCDC90B protein.

To create an overexpression of the T1 isoform we infected MCF-10A cells with retrovirus packaged from the pQCXIH vector containing the T1-CCDC90B coding sequence. The PQCXIH vector contains a CMW promoter, multiple cloning site (MCS), internal ribosome entry site (IRES), and the hygromycin resistance proteins (figure 2). The T1-CCDC90B flag tagged coding sequence is in the MCS and one set of cells were infected with the vector containing this sequence (T1-CCDC90B overproducer cells) while the other set of cells were infected with the vector without the T1-CCDC90B ORF (control cells). The vector also contains a strong CMV promoter upstream of the MCS (which contains the T1-CCDC90B transcript) and hygromycin resistance gene. The internal ribosome entry site (IRES) allows multiple proteins to be translated from the same mRNA sequence, ensuring that the hygromycin resistance and T1-CCDC90B proteins are both translated in tandem. The T1-CCDC90B-Flag expression is significantly higher than the endogenous CCDC90B protein; however, they need to be cultured in hygromycin (50 ug/mL) in order to keep the expression of T1-CCDC90B protein stable (figure 2). However, when conducting our experiments to get a better physiological sense of what effects the protein had on the mitochondria the Flag tag was later removed.
To knockdown the CCDC90B protein, we infected MCF-10A cells with lentivirus packaged from a shRNA construct in the pIko.1 puro vector. The shRNA we used targets the CCDC90B gene in the 3’ UTR region and targets all functional isoforms. We were able to achieve efficient and stable knockdown of CCDC90B RNA and protein (figure 3).

2.1 T1-CCDC90B is located in the mitochondria

To determine the location of T1-CCDC90B in MCF10A cells, we performed an immunofluorescence assay on the MCF-10A overexpression. We seeded both the T1-CCDC90B overexpression cells along with the control cells in chamber slides with growth media containing hygromycin. The cells were incubated for 72 hours before they were stained and then fixed (see materials and methods for procedure). The cells were stained for MitoTracker Orange (a mitochondrial membrane potential dependent mitochondrial stainer) and probed for the CCDC90B protein. Using a confocal microscope, we were able to take Z-stack 3D images of the cells in addition to normal 2D images. Our control cells did not have enough steady state CCDC90B protein to get an idea of where the protein is located; however, using the T1-CCDC90B overproducer cells, we show that the protein is localized in the mitochondria (figure 4). Analysis of the image showed that T1-CCDC90B protein perfectly co-localized with the MitoTracker orange staining; thus, areas of increased MitoTracker Orange staining intensity correlated to an increase in T1-CCDC90B protein staining. Therefore, we can conclude that T1-
CCDC90B is localized in the mitochondria and that the protein may play a role in the mitochondria.

2.2 T1-CCDC90B effect on COX1

In addition to staining the cells with MitoTracker orange, we probed the cells with other mitochondrial markers like COX1 (protein subunit in complex IV of ETC) along with CCDC90B. As expected we see there is colocalization between the CCDC90B protein and COX I; however, an interesting result we saw was that compared to the control cells the T1-CCDC90B overproduced cells had increased COX I staining (figure 5). COX1 is a critical protein in complex IV of the ETC and by analyzing both cell lines through IF there is no difference in the percentage of cells that stained positive for COX1, but the T1-CCDC90B overproducer cells appeared to have more cells that had a “high” COX1 signal. For this qualitative result, we determined “high” COX1 by using the Imagej software to count the number of cells that had a certain level of COX1 staining intensity. As a result of this analysis, we saw that despite having a similar percentage of cells that stained positive for COX1, about 45% of the T1-CCDC90B overproducer cell line had “high” COX1 staining compared to the control cell line in which around 12% of the cells had “high” COX1 staining (figure 5). After seeing this drastic difference in COX1 staining intensity, we tested if the T1-CCDC90B overexpression led to an increase in COX1 protein expression. We saw that the COX1 protein expression did in fact increase in the T1-CCDC90B overexpression cell line when compared to the control
After seeing an increase in COX1 protein we looked to see what effect this can have on the cell.

Because COX1 is an integral subunit in complex IV of the ETC, we were curious to see if there was a correlation between increased COX1 staining and active mitochondria. Similar to the previous experiment, the cells grew for 72 hours and were stained with MitoTracker orange before being fixed and probed for COX1. We were then able to use the Imagej program to quantify the exact staining intensity of each channel. By examining the raw data, we can see that there are a few differences between the T1-CCDC90B overproducer cells and the control cells (figure 6). When observing the raw MitoTracker orange staining intensities, the population of cells in both cell lines had similar intensities. This suggests that T1-CCDC90B did not have an effect on active mitochondria (figure 6). But similar to the previous experiment, it appears that the population of T1-CCDC90B overproducer cells still had a higher COX1 staining when compared to the control (figure 6). By taking each individual cell’s COX1 intensity and normalizing it to its MitoTracker orange intensity we get the ratio of each cell’s COX1 per active mitochondria. Through multiple experiments, we can conclude that there is a significant difference in the amount of COX1 per active mitochondria as the T1-CCDC90B overproducer cells had over three times the ratio when compared to the control cells (figure 6).

Because hygromycin is a drug, we wanted to see if this change was due to hygromycin or if it was due to the overexpression of T1-CCDC90B protein and if it was due to the protein could the effects be reversed. From previous experiments, it is known that for T1-
CCDC90B overproduction expression to be stable it needs to be cultured in hygromycin; therefore, we looked for a way to lower the T1-CCDC90B protein in the cell line to see if we can reverse the effect on COX1. To reduce the T1-CCDC90B levels, we looked for an optimal time in which the removal of hygromycin reduced T1-CCDC90B protein in the overproducer cell line. We noticed that keeping the cells off hygromycin for 48 hours was the optimal time in order to reduce T1-CCDC90B levels (see material and procedure for step by step protocol). Using this hygromycin removal protocol, we saw the T1-CCDC90B cells in which hygromycin was removed lost a significant amount (4 times less) of T1-CCDC90B protein while the cells that had hygromycin kept in still maintained normal T1-CCDC90B staining (figure 8). Since we were able to lower the CCDC90B protein using this protocol, we wanted to see if we can reverse the COX1 effect seen earlier. We saw that in the T1-CCDC90B overproducer cells that had hygromycin kept in still maintained a high COX1/MitoTracker orange ratio when compared to the control. However, in the T1-CCDC90B overproducer cells where hygromycin was removed the COX1/MitoTracker orange ratio dropped to the control level (figure 9). Therefore, we saw that removing hygromycin and lowering T1-CCDC90B protein caused a reverse in the phenotype by lowering COX1 staining levels similar to the control cell line without affecting the active mitochondria. This led us to the conclusion that the increase in COX1 staining is not due to hygromycin, but rather the increase in T1-CCDC90B protein and lowering the protein will reverse this effect.

Based on these results CCDC90B appears to play a role in the COX1 protein, but the exact role it plays is not yet known. Previous studies have shown that CCDC90B has a strong interaction with CCDC90A via immunoprecipitation (Tomar et al, 2016) and that
CCDC90A may play a role in COX assembly (Paupe et al., 2015). COX has subunits that are mitochondrial encoded such as COX1 and COX2. Interestingly enough, there are results that suggest that CCDC90A stabilizes COX2 and may, in fact, chaperone and stabilize COX2 into the COX complex (Paupe et al, 2015). T1-CCDC90B may play a similar role as the CCDC90B-COX1 interaction may stabilize COX1 which increases the steady state of COX1 in the cell.

2.3 T1-CCDC90B Protein Affects the Mitochondrial Compartment Size

In the previous experiments that I have conducted we saw an increase in COX1, but there was no difference in the active mitochondria staining. Our next step was to see if there was a difference in mitochondrial potential per total mitochondrial compartment size. To achieve this we stained the cells with TMRE (mitochondrial potential dependent mitochondrial stainer) and MitoTracker green (total mitochondrial stainer not dependent on potential) before performing live cell imaging (see methods and procedure). We had to perform live cell imaging as both the TMRE and MitoTracker green dyes cannot be fixed. Similar to previous experiments, we seeded the cells and incubated for 72 hours before staining and performed live cell imaging via the confocal microscope (figure 7). By observing the raw intensity values between the populations of the two cell lines the TMRE intensity is not significantly different, which is similar to what we saw in previous experiments (figure 7). However, when observing the MitoTracker green staining intensity a significant population of cells in the T1-CCDC90B overproducer cell line had a lower MitoTracker green staining intensity when compared to the control cell line.
To obtain the membrane potential per total mitochondrial compartment size for each individual cell, we took the TMRE staining intensity of each cell and divided it by the MitoTracker green staining intensity for the same cell. This ratio is the MP/MC (mitochondrial potential/mitochondrial compartment size). Upon multiple experiments, we concluded that the MP/MC in the T1-CCDC90B overexpression cells is roughly twice that of control cell line (figure 7). Like the increase in COX1 signal, we wanted to test if this phenotype was reversible if we lower the T1-CCDC90B levels in the cell. In order to do this, we conducted our hygromycin removal protocol and stained the cells with MitoTracker green and TMRE (figure 10). Similar to all the previous experiments, when analyzing the raw staining intensity values of TMRE, there is no difference in staining intensity between either cell line under both conditions (hygromycin kept in or hygromycin removed) (figure 10). We also see little difference between the two conditions in the control cell line when comparing MitoTracker green staining intensity (figure 10). These results suggest that hygromycin has no effect on either the TMRE or MitoTracker green staining. Similar to the 72-hour experiment conducted previously, the T1-CCDC90B overexpression cells in which hygromycin was kept in still had a low MitoTracker green staining intensity; but in the T1-CCDC90B overproducer cells in which hygromycin was removed, the MitoTracker green staining intensity was similar to both the control cell lines (figure 10). Thus, lowering the T1-CCDC90B protein by removing hygromycin in the overproducer cells reversed the effect seen before by increasing the MitoTracker green staining intensity close to the control levels. Upon multiple experiments and taking MP/MC of the two cell lines in both conditions, we conclude that hygromycin has no effect on the ratio and that cell line with high T1-
CCDC90B have roughly twice the MP/MC than cell lines with low T1-CCDC90B (figure 10). It should be noted that the MP/MC ratios gathered in the hygromycin removal experiment mimic the results seen in the 72 hour experiment as both cell lines which had high T1-CCDC90B had roughly 2 fold increase in the MP/MC ratio when compared to cells with low T1-CCDC90B cells. Based on these results, we can conclude that overexpression of T1-CCDC90B causes an increase in the mitochondrial potential per unit mitochondria compartment size and this is due to a decrease in compartment size. We were also able to conclude that lowering the T1-CCDC90B protein in the cells reverses this effect.

Because MitoTracker green is a dye that stains for total mitochondrial compartment size we wanted to see if the mitochondrial proteins changes as well. Out of the key mitochondrial markers we probed for, there was no significant difference in the proteins expressed in the mitochondria (figure 7). Because there are thousands of proteins in the mitochondria, we cannot rule out a change in mitochondrial mass. However, we continued under the hypothesis that the change is just strictly mitochondrial compartment size.

We wanted to test this phenomenon and see if it was physiological so we tested the effect on CCDC90B knockdown cells (sh6). We conducted the same experimental procedure and imaged the live cells after staining for TMRE and MitoTracker green (figure 11). Similar to previous experiments, we see no significant difference in TMRE staining intensity, but still get a significant shift in the MitoTracker green staining intensity (figure 11). The MitoTracker green staining intensity in the sh6 cells
(CCDC90B knockdown) cells had a higher MitoTracker staining intensity compared to the control. Upon multiple experimental repeats, we saw that the MP/MC for each cell in the Sh6 cell line had roughly half the ratio when compared to the control (figure 11). So in the T1-CCDC90B overproducer cells we saw that the MP/MC had twice the ratio when compared to control, but in the CCDC90B knockdown cell line the ratio is half. In both of these cases, the difference in the ratio is due to the MitoTracker green staining or the mitochondrial compartment size. This suggests that CCDC90B does have a physiological effect on the mitochondrial compartment size.

A possible reason for the significant change in MitoTracker green staining is that CCDC90B may play a role in the activity of the ion channels which affects the matrix volume. As stated in 1.6, ions play an important role in mitochondrial matrix volume regulation. If CCDC90B plays a role in increasing the activity of an ion channel in which a net efflux of ions occurs, it would count for the lower MitoTracker green staining intensity seen when T1-CCDC90B is overproduced and a decrease in staining when CCDC90B is knocked down. If the ion flow is changing, it is also vital to detect any secondary effects that may occur as a change in both the volume and ion concentrations have many secondary effects on the mitochondria and the overall cell. As stated previously, we did not see an increase in mitochondria mass when looking at some key mitochondrial proteins, however, this possibility cannot be ruled out. A future experiment would be to isolate the mitochondria and see if the increase in compartment size also leads to an increase in mitochondrial mass.
The difference seen in both the COX1 and the compartment size of the mitochondria can have various effects on the cell. COX1 is one of the vital subunits in the COX complex and the instability or decrease of COX1 leads to a deficiency of the COX complex. COX1 is one of the subunits directly responsible for the redox reactivity of complex IV (Diaz et al, 2010). Since we see an increase in the steady state of COX1 due to T1-CCDC90B overexpression it is possible we may see an increase in the complex IV activity. Therefore, an additional future experiment is to directly test the complex IV activity of the cell as well as the oxygen consumption rate to see if the increase in COX1 correlates with an increase in oxygen consumption and complex IV activity.
Figure 1. Human CCDC90B gene and transcripts and the coding sequence for T1-CCDC90B. (A) The three functional isoforms for human CCDC90B protein all three isoforms have the same 9 exons. (B) The T1-CCDC90B protein has a coding sequence consisted of 254 amino acids and contains a mitochondrion targeting sequence and a transmembrane helix. The T1-CCDC90B transcript also has an evolutionally conserved DUF 1640 which contains the Coiled-coil domain sequence.
Figure 2. Overexpression of T1-CCDC90B protein in MCF-10A cells. (A) PQCXIH hygromycin resistance vector infected in MCF-10A cells. The PQCXIH vector contains a CMV promoter upstream from the T1-CCDC90B coding sequence, IRES and hygromycin resistance coding sequence. The IRES site allows multiple proteins to be translated in same mRNA. (B) For the western blot samples were collected 72 hours after seeding. As both control cells (vector containing cells without T1-CCCDC90B transcript) and T1-CCDC90B overproducer cells were passed they were passed in conditions that lacked hygromycin (-) or contained 50 ug/mL of hygromycin (+).
Figure 3. **Knockdown of CCDC90B protein in MCF-10A cells** (A) pLKO.1 puromycin vector infected into MCF10A cells. The shRNA gets transcribed and is downstream from the U6 promoter. Once shRNA gets transcribed becomes mature siRNA which can target the 3’UTR region of CCDC90B protein. (B) Western Blot with samples collected 72 hours after seeding. The sh6 cell line (CCDC knockdown cells) efficiently knockdown CCDC90B when compared to control and wild type.
Figure 4. **T1-CCDC90B is localized in the mitochondria.** T1-CCDC90B overexpression cells grown in growth media containing hygromycin (50 μg/mL) for 72 hours before stained with MitoTracker orange (100 nM) and fixed with 4% PFA. The cells were then probed for CCDC90B protein and imaged using confocal microscope and image taken at 100x. The merged image perfect colocalization between MitoTracker orange (red) and CCDC90B(green).
Figure 5. **T1-CCDC90B overproducer cells have an increased COX1 staining when compared to control.** Both T1-CCDC90B overproducer cells and control cells were grown in growth media containing hygromycin (50 μg/mL) for 72 hours before fixed with 4% PFA. (A) The cells were probed for CCDC90B and COX1 and imaged using confocal microscope at 60x. (B) Cell count of number of cells which had a high COX1 signal.
Figure 6a. **T1-CCDC90B overproducer cells have increased COX1 protein but has no effect on active mitochondria.** (A) both T1-90B overproducer and control cells were grown in growth media with hygromycin (50 ug/mL) for 72 hours the cells were stained for MitoTracker orange (100 nM) (red) and fixed with 4% PFA. The cells were then probed for COX1 (green) and imaged using confocal microscope at 60x. (B) The raw intensity values of COX1 and MitoTracker orange staining intensity for each cell.
Figure 6b. T1-CCDC90B overproducer cells have increased COX1 protein but has no effect on active mitochondria. (C) Upon multiple experimental repeats (n=3) the ratio of COX1 staining per active mitochondria in both cell lines. (D) Western blot probing for COX1 for T1-CCDC90B overproducer cells and the vector cells collected at 72 hours grown in hygromycin (50 μg/mL)
Figure 7. **T1-CCDC90B overproducer cells have a higher mitochondrial potential per total mitochondrial compartment size.** (A) Both T1-90B overproducer and control cells were grown in growth media with hygromycin (50 μg/mL) for 72 hours the cells were stained for TMRE (100 nM) (red) and MitoTracker green (100 nM) (green) before live cell imaging at 60x. (B) Raw intensity values of TMRE and MitoTracker green staining of each individual cell between the two cell lines. (C) Upon multiple repeats (n=3) the ratio of each individual cell’s mitochondrial potential over its total mitochondrial compartment size (TMRE/MitoTracker green). (D) Western blot of T1-CCDC90b overproducer cells and vector cells collected at 72 hours grown in hygromycin.
Figure 8. **Removal of hygromycin caused a decrease in T1-CCDC90B protein.** (A) Followed hygromycin removal protocol (see materials and methods) and fixed the cells with 4% PFA. Probed for CCDC90B (green) in the T1-CCDC90B cells in which hygromycin was removed and in the cells in which hygromycin was kept in and imaged at 40x. (B) The percentage of cells which were positive for CCDC90B staining between the two conditions.
Figure 9. Decreasing CCDC90B protein caused a reverse in COX1 staining. (A) Followed hygromycin removal protocol and stained the cells with MitoTracker orange (100 nM) (red) before fixing the cells in 4% PFA. The cells were then probed for COX1 (green) before imaging at 60x via confocal microscope. (B) The ratio of each individual cell’s COX1 staining per active mitochondria
Figure 10a. **Reduction of T1-CCDC90B restored MitoTracker green staining.** Followed hygromycin removal protocol (+/+ cells are kept in hygromycin and +/- cells have hygromycin removed). The cells were live cell imaged and were stained with MitoTracker green (100 nM) (green) and TMRE (100 nM) (red). (A) MitoTracker green staining is shown of both cell lines in both conditions. (B) Raw MitoTracker green intensity values of the two cell lines in both conditions.
**Figure 10b. Reduction of T1-CCDC90B restored MitoTracker green staining.** Followed hygromycin removal protocol (+/+ cells are kept in hygromycin and +/- cells have hygromycin removed). The cells were live cell imaged and were stained with MitoTracker green (100 NM) (green) and TMRE (100 nM) (red). (C) Merged image of TMRE (red) and MitoTracker green (green) stained cells. (D) Raw TMRE and MitoTracker green staining intensity values of individual cells between the two cell lines in both conditions. (E) Upon multiple repeats (n=3) the ratio of each individual cell’s mitochondrial potential over its total mitochondrial compartment size.
Knockdown of CCDC90B caused an increase in MitoTracker green staining. Both pLKO vector cells and sh6 cells were grown in similar conditions to hygromycin removal protocol sans hygromycin. The cells were stained with TMRE (100 nM) (red) and MitoTracker green (100nM) (green) before imaged at 60x via confocal microscope (A) MitoTracker green staining of both cell lines. (B) Average MitoTracker green intensity values for each cell line.
Figure 11b. Knockdown of CCDC90B caused an increase in MitoTracker green staining. Both pLKO vector cells and sh6 cells were grown in similar conditions to hygromycin removal protocol sans hygromycin. The cells were stained with TMRE (100 nM) (red) and MitoTracker green (100nM) (green) before imaged at 60x via confocal microscope (C) Both cell lines are stained with TMRE and MitoTracker green (D) Raw intensity values of TMRE and MitoTracker green staining of each individual cell between the two cell lines. (E) Upon multiple repeats (n=3) the ratio of each individual cell’s mitochondrial potential over its total mitochondrial compartment size (TMRE/MitoTracker green).
III. Materials and Methods
4.1 Cell culture

MCF-10A cells were maintained in DMEM:F-12 growth media (cat # 11330-032) supplemented with 5% Horse Serum and a final concentration of 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10ug/mL of insulin and 1% penicillin and streptomycin. The cells were grown to roughly 90% confluence in 6 cm plates before cells were trypsinized for an assay or passage. For PQCXIH vector containing cells (T1-CCDC90B overproducer and control cells) 50 ug/mL hygromycin added

4.2 Hygromycin Removal Protocol

1) Seed 2 sets of PQCXIH vector congaing cells in hygromycin for experiment
2) Allow the cells to grow in growth media with hygromycin for 48 hours
3) 48 hours after seeding aspirate media
4) Wash the cells with regular MCF-10A growth media 3 times
5) In one set of PQCXIH vector congaing cells add growth media without hygromycin.
   In the other set of PQCXIH vector containg cells add grpwth media with hygromycin
6) Allow cells to grow for an additional 48 hours (96 hours total)
7) Perform assay
4.3 Cell Immunofluorescence and cell staining protocol

4.3i: 72 hour live staining

1) seed 30,000 cells into Nunc Lab Tek II 8 chamber slide wells
2) incubate for 72 hours
3) after 72 hours aspirate media and stain cells with dyes (see dye concentration list) diluted in normal growth media for 25 minutes
4) After 25 minutes aspirate the media with the dye and wash with 1x PBS
5) image using confocal microscope

4.3ii: Hygromycin removal live staining

1) Seed 20,000 cells into Nunc Lab Tek II 8 chamber slide wells
2) Follow hygromycin removal protocol (4.2)
3) after 96 hours aspirate media and stain cells with dyes (see dye concentration list) diluted in normal growth media for 25 minutes
4) After 25 minutes aspirate the media with the dye and wash with 1x PBS
5) image using confocal microscope

4.3iii: 72 hours cell Immunofluorescence

1) Seed 30,000 cells into Nunc Lab Tek II 8 chamber slide wells
2) Incubate for 72 hours

3) **Optional step depending on experiment**: aspirate media and stain with MitoTracker orange (see dye concentration list) diluted in normal growth media for 25 minutes

4) Aspirate media and fix cells with 4% PFA for 10 minutes in room temperature

5) Wash cells with PBS 3 times for 5 minutes each wash

6) Permeabilize cells with .1% Triton-X-100 in PBS for 15 minutes

7) Wash cells with PBS 3 times for 5 minutes each wash

8) Block cells with 5% BSA in PBS (blocking solution) for 1 hr in in room temperature

9) Wash cells with PBS 3 times for 5 minutes each wash

10) Add diluted primary antibody of interest (see antibody concentration list) into blocking solution to cells and incubate in room temperature for 2 hours

11) Wash cells with PBST 3 times for 5 minutes each wash

12) Add diluted secondary antibody of interest and DAPI (see antibody and dye concentration list) into blocking solution to cells and incubate in room temperature for 1 hour

13) Wash cells with PBS 3 times for 5 minutes each wash

14) Image using confocal microscope

**4.4iv: Hygromycin removal Immunofluorescence**

1) Seed 20,000 cells into Nunc Lab Tek II 8 chamber slide wells

2) Follow hygromycin removal protocol (4.2)
3) **Optional step depending on experiment**: aspirate media and stain with MitoTracker orange (see dye concentration list) diluted in normal growth media for 25 minutes

4) Aspirate media and fix cells with 4% PFA for 10 minutes in room temperature

5) Wash cells with PBS 3 times for 5 minutes each wash

6) Permeabilize cells with .1% Triton-X-100 in PBS for 15 minutes

7) Wash cells with PBS 3 times for 5 minutes each wash

8) Block cells with 5% BSA in PBS (blocking solution) for 1 hr in in room temperature

9) Wash cells with PBS 3 times for 5 minutes each wash

10) Add diluted primary antibody of interest (see antibody concentration list) into blocking solution to cells and incubate in room temperature for 2 hours

11) Wash cells with PBST 3 times for 5 minutes each wash

12) Add diluted secondary antibody of interest and DAPI (see antibody and dye concentration list) into blocking solution to cells and incubate in room temperature for 1 hour

13) Wash cells with PBS 3 times for 5 minutes each wash

14) Image using confocal microscope

**Antibody concentration:**

COX I (life technology): 1:500 dilution

Cox IV (genetex): 1:1000 dilution

CCDC90B (novex): 1:500 dilution

Anti-FLAG (sigma): 1:500 dilution
Dye Concentration:

TMRE (ab113852): 100 nM

MitoTracker Orange (M7510): 100 nM

MitoTracker Green (M7514): 100 nM
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