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Chelsea Lee March 23, 2018

Interactions Between Mitochondrial Transporters and the Protein Synthesis Machinery in 22q11.2 Deletion Syndrome

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Abstract

Interactions Between Mitochondrial Transporters and the Protein Synthesis Machinery in 22q11.2 Deletion Syndrome

By Chelsea Lee

Schizophrenia is a chronic and complex neuropsychiatric disorder. Schizophrenia's polygenic nature makes it difficult to study and pinpoint causative disease mechanisms. To circumvent these challenges, our lab chose to focus on 22q11.2 Deletion Syndrome (22q11.2DS). 22q11.2DS is the highest genetic risk factor for schizophrenia aside from having two parents or an identical twin diagnosed with schizophrenia. Our lab used quantitative proteomic analysis to compare fibroblasts from 22q11.2DS patients and their unaffected family members. The proteome revealed that the mitochondria was one of the central organelles affected in 22q11.2DS. This lead our lab to propose the following overarching hypothesis: *defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome contribute to behavioral and synaptic phenotypes shared by schizophrenia and other neurodevelopmental disorders.* The proteome showed hits on the Electron Transport Chain and the inner mitochondrial matrix. Immunoblots showed altered levels of SLC25A1 (a mitochondrial citrate transporter) and proteins encoding for mitochondrial ribosome subunits in 22q11.2DS patients. The SLC25A1 gene is found in the core deletion region of 22q11.2DS. These findings were central to my thesis, leading to the hypothesis that: ***SLC25A1 null cells possess altered mitochondrial protein synthesis leaving them more susceptible to mitochondrial ribosome protein synthesis inhibiting drugs***. To test this, I used Agilent Seahorse Mitochondrial Stress Test assays to measure mitochondrial respiration rates in HAP1 SLC25A1 KO cells and HAP1 control cells treated with varying antibiotics that inhibit mitochondrial ribosome protein synthesis or proteostasis (minocycline, doxycycline, chloramphenicol, linezolid, and actinonin). I predicted that SLC25A1 null cells should have

differential susceptibility to agents that selectively block mitochondrial ribosome protein synthesis as compared to wild type cells. Antibiotics used varied in structural chemistry and mechanisms of action. In cells lacking SLC25A1, I found that treatment with antibiotics targeting the mitochondrial ribosome led to a decrease in oxygen consumption rates. HAP1 control cells showed no decrease in oxygen consumption rates. To ensure that the decrease in mitochondrial respiration in SLC25A1 null cells was due to mitochondrial ribosome dysfunction, cells were also treated with carbenicillin, a beta lactamase inhibitor. Neither the HAP1 control cells or HAP1 SLC25A1 KO cells showed a significant decrease in mitochondrial respiration when treated with carbenicillin. These results support my hypothesis that SLC25A1 null cells possess altered mitochondrial protein synthesis and are more susceptible to drugs inhibiting mitochondrial ribosome protein synthesis.

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Table of Contents

Introduction……1
Methods……18
Results……..,23
Discussion……,35
References……42

CHAPTER 1: INTRODUCTION

Overview

Schizophrenia is a chronic and complex neuropsychiatric disorder that affects an estimated 1.1% of the adult population in the United States (Regier *et al.*, 1993). Manifestation of the disease usually occurs in early adulthood. Despite the prevalence of schizophrenia, current treatments are limited to psychotherapy and antipsychotic drugs. Schizophrenia has a polygenic nature that has created barriers to fully understanding the mechanisms of the disease. With genome-wide association studies (GWAS) and exon sequencing studies revealing over 100 specific common risk loci and at least 11 rare risk alleles, the mutational target for schizophrenia may involve thousands of genes (Kavanagh *et al.*, 2015). With schizophrenia's complex nature providing challenges to pinpointing causative disease mechanisms, researchers must turn their focus to well-defined patient populations with strong genetic risk factors for schizophrenia that mimic the polygenicity of this disorder.

Chromosome 22q11.2 deletion syndrome (22q11.2DS) – often clinically referred to as DiGeorge Syndrome – is a genetic syndrome that arises from a hemizygous 1.5 or 3 Mb deletion on chromosome 22. Despite the deletion size, the core phenotypes in 22q11.2 DS are similar in patients carrying the 1.5 or the 3 Mb deletion (McDonald-McGinn *et al.*, 2015). The complexity of this microdeletion is highlighted by the observation that the 3 Mb mutation reduces by half the gene dosage of 63 genes, 46 of which encode proteins (Figure 1). This complexity has been a hurdle to define molecular mechanisms that could explain the systemic and neurodevelopmental symptoms observed in 22q11 DS patients. With 25-30% of 22q11.2DS patients developing schizophrenia and 22q11.2DS accounting for 1-2% of sporadic schizophrenia cases in the general population, 22q11.2DS is the strongest known genetic risk factor for schizophrenia (Gothelf *et al.*, 2004; Debbane *et al.*, 2006; Green *et al.*, 2009; Fung *et al.*, 2010; Karayiorgou *et al.*, 2010; Drew

et al., 2011). 22q11.2DS offers an ideal model to study schizophrenia, that accounts for the complexity of schizophrenia as a polygenic disorder.

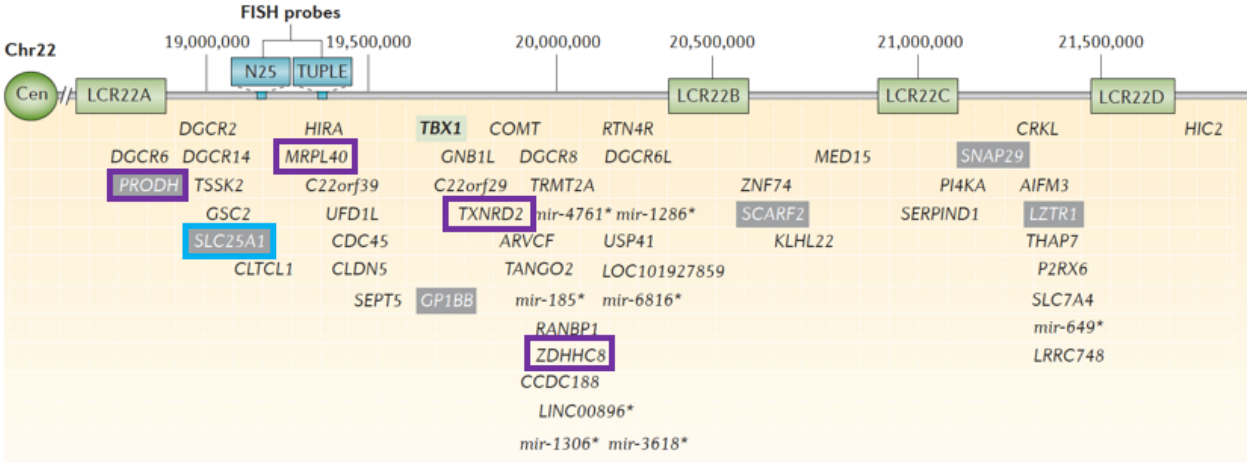

Figure 1. Genes located on in the 22q11.2 deletion region (McDonald-McGinn *et al.*, 2015). Genes associated with the mitochondria are highlighted.

While little is known about which genes in the 22q11.2 segment contribute to 22q11DS's patient psychiatric phenotypic traits and the cellular and molecular basis of these, our laboratory developed a human pedigree-based quantitative mass spectrometry strategy named *genealogical proteomics* to aid in addressing the complexity associated with 22q11.2DS's complex polygenic nature. This strategy allowed us to identify cellular and molecular mechanisms downstream of the whole genetic defect rather than to study isolated genes within the 22q11 chromosomal segment. Fibroblasts were taken from patients with both 22q11.2DS and diagnosed psychosis and their unaffected family members. The proteome of fibroblasts was quantified and compared those of unaffected family members and patients with 22q11.2DS and psychosis. We identified cellular and molecular pathways within the Ch22q11-sensitive proteome that comprehensively encompass the effect of this complex 22q11.2 deletion in cellular mechanisms. Analysis of the 22q11 proteome showed an enrichment of mitochondrial proteins whose expression was sensitive to the 22q11 deletion. The significant enrichment of these mitochondrial hits in the 22q11.2DS patient

cells provides evidence that the mitochondria is a central affected organelle in 22q11.2DS. This finding lead to the following *overarching hypothesis for our laboratory:*

> *defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome contribute to behavioral and synaptic phenotypes shared by schizophrenia and other neurodevelopmental disorders.* This hypothesis and its foundations provide the framework for my honor thesis.

Central to my thesis, the genealogical ontologies derived from the 22q11.2 proteome showed hits on the Electron Transport Chain (ETC) and the inner mitochondrial matrix. Immunoblots showed altered levels of SLC25A1 (a mitochondrial citrate transporter) and mitochondrial ribosome subunits in 22q11.2DS patients. As SLC25A1 is in the core microdeletions regions of 22q11.2DS,

> ***I hypothesize that SLC25A1 null cells possess altered mitochondrial protein synthesis leaving them more susceptible to mitochondrial ribosome protein synthesis inhibiting drugs.***

To test this hypothesis, I will measure mitochondrial respiration levels in cells lacking SLC25A1 as a global output of mitochondrial functions which is susceptible to perturbations of mitochondrial transporter activity and/or protein synthesis. In addition, I will also measure the mitochondrial respiration levels in SLC25A1 null cells that have been treated with various antibiotics that aim to inhibit mitochondrial protein synthesis to assess if genetic defects in SLC25A1 render cells more susceptible to drugs that inhibit mitochondrial protein synthesis.

In the following sections, I will go into further details regarding the relationship between 22q11.2DS and schizophrenia. I will also further describe etiology and phenotypes associated with 22q11.2DS. I will also discuss how our genealogical proteomic study sheds light onto how

schizophrenia and 22q11DS are related. Finally, I will briefly describe my project and hypothesis for my thesis.

Significance

An estimated 4.2% of the adult population in the United States experience serious mental illness each year. Among those are the 1.1% of adults who are affected by schizophrenia (Regier *et al.*, 1993). Schizophrenia is a chronic, severe neuropsychiatric disorder that can manifest itself in a variety of symptoms. Symptoms are generally characterized as either "positive" or "negative." Positive symptoms – such as hallucinations and delusions – are categorized as symptoms that are not present in a neurotypical person. Negative symptoms – such as apathy, social withdrawal, and low affect – are symptoms that are present in neurotypical people but lacking in those with schizophrenia. In the field of schizophrenia research, various studies and techniques have been used to further understand the disorder. Despite family, twin, and adoptions studies and the use of large scale genome-wide association studies (GWAS) to identify genetic risk factors of schizophrenia, the polygenic nature of the disorder has brought challenges to pinpointing the precise targets (Ming & Weinberger, 2017). With GWAS and exon sequencing studies revealing over 100 specific common risk loci and at least 11 rare risk alleles, the mutational target for schizophrenia may involve thousands of genes (Kavanagh *et al.*, 2015).

Despite difficulties due to schizophrenia's polygenic nature, it has been discovered that there is a high correlation between schizophrenia and the patient population of Chromosome 22q11.2 Deletion Syndrome (22q11.2DS), with 25-30% of patients with 22q11.2DS developing schizophrenia (Gothelf *et al.*, 2004; Green *et al.*, 2009; Fung *et al.*, 2010; Drew *et al.*, 2011). Furthermore, subthreshold symptoms indicative of psychosis are seen in one-third to one-half of

children diagnosed with 22q11.2DS (Feinstein *et al.*, 2002; Baker & Skuse, 2005; Debbane *et al.*, 2006; Drew *et al.*, 2011). In addition, individuals diagnosed with 22q11.2DS account for 1-2% of sporadic schizophrenia cases in the general population (Karayiorgou *et al.*, 2010; Drew *et al.*, 2011). From these statistics, we see the extent to which schizophrenia is enriched in the 22q11.2DS patient population. 22q11.2DS provides an ideal model system to further explore the unknown cellular mechanisms underlying schizophrenia.

Chromosome 22q11.2 Deletion Syndrome and Schizophrenia

Chromosome 22q11DS – also known as DiGeorge Syndrome – is the most common chromosomal microdeletion, presenting in an estimated 1 in 4000 live births (Karayiorgou *et al.*, 2010; McDonald-McGinn *et al.*, 2015; Devaraju & Zakharenko, 2017). Individuals with 22q11.2DS have either a 1.5 or 3 Mb deletion on the long arm of chromosome 22. This deletion is found to be *de novo* in 90-95% of 22q11.2 patients (McDonald-McGinn *et al.*, 2015). An estimated 90% of 22q11.2DS microdeletions are 3Mb, while ~8% are 1.5 Mb deletions. Previous studies have indicated that the 3 Mb deletion is typically mediated by low copy repeat non-homologous recombination (Edelmann *et al.*, 1999; Michaelovsky *et al.*, 2012). Despite the deletion size, the core phenotypes of the syndrome are present and there is no correlation between the size of the deletion and the severity of the disease phenotypes (Drew *et al.*, 2011). The 22q11.2 region contains several large blocks of low copy repeats (LCRs) and segmental duplications, making the deleted region in 22q11.2DS one of the most structurally complex areas of the human genome (Edelmann *et al.*, 1999; Michaelovsky *et al.*, 2012; McDonald-McGinn *et al.*, 2015).

In addition to an increased risk of schizophrenia, patients with 22q11.2DS also have a higher chance of presenting with Autism Spectrum Disorder (ASD), Attention Deficit

Hyperactivity Disorder (ADHD), anxiety, and language learning disabilities (Gothelf *et al.*, 2004; Bassett *et al.*, 2005; Antshel *et al.*, 2007; Niklasson *et al.*, 2009). Among children referred for clinical laboratory testing for suspicion of developmental disabilities, 1 per 169 were diagnosed with 22q11.2DS; however, 22q11.2DS is not found in samples of seemingly healthy children (Kaminsky *et al.*, 2011). Historically, 22q11.2DS has been diagnosed using fluorescence in situ hybridization (FISH), but more recently other methods such as multiplex ligation-dependent probe amplification (MLPA) or chromosomal microarrays are being used to diagnosed 22q11.2DS (Vorstman *et al.*, 2006; Sorensen *et al.*, 2010).

In addition to the neurodevelopmental disorders described above, chromosome 22q11.2DS manifests itself in a variety of ways across multiple organ systems. These include conotruncal cardiovascular malformations, craniofacial malformations, velopharyngeal insufficiency, thymus defects and defective plasma calcium homeostasis (Meechan et al., 2015). As mentioned previously, not all patients with 22q11.2DS present with all manifestations. These protean manifestations have prompted the question of what are the cellular and molecular mechanisms that leads to these diverse problems. However, the complexity of the mutation has limited our progress in understanding disease pathogenesis.

Despite the complexity of both the 22q11.2DS and schizophrenia, it has been discovered that there is a high correlation between schizophrenia and the patient population of Chromosome 22q11.2 Deletion Syndrome (22q11.2DS), with 25-30% of patients with 22q11.2DS developing schizophrenia (Gothelf *et al.*, 2004; Green *et al.*, 2009; Fung *et al.*, 2010; Drew *et al.*, 2011). Furthermore, it has been discovered that more than half of adolescents with 22q11.2DS experience schizotypal traits and transient psychotic experiences (Baker & Skuse, 2005). These findings place patients with 22q11.2DS as the third most at risk population for schizophrenia, only behind those

with both parents or an identical twin with schizophrenia (Debbane *et al.*, 2006). Some studies have shown that children with 22q11.2DS who show decreased verbal skills, poor social functioning, and cognitive deficits in the executive functioning domain – which include abstraction, mental flexibility, attention and working memory – may be more likely to develop schizotypal behavior, as opposed to 22q11.2DS patients where psychosis does not manifest (Baker & Skuse, 2005; Debbane *et al.*, 2006; Weinberger *et al.*, 2016). While previous literature has shown a correlation between cognitive defects and the presence of psychosis in 22q11.2Ds patients, the underlying biological mechanisms that lead to this is still unclear. If we are able to further understand why 22q11.2DS such drastically increases the risk for schizophrenia, we may be able to better understand the biological mechanisms that lead to schizophrenia.

Past Approaches and Challenges

22q11DS expands either a 1.5 to 3.0 Mb chromosomal segment – the hemizygous deletion yields a heterogeneous clinical presentation yet the core phenotypes in 22q11.2 DS are similar in patients carrying the 1.5 or the 3 Mb deletion (McDonald-McGinn *et al.*, 2015). The complexity of this microdeletion is highlighted by the observation that the 3 Mb mutation reduces by half the gene dosage of 63 genes, 46 of which encode proteins. This complexity has been a hurdle to define molecular mechanisms that could explain the systemic and neurodevelopmental symptoms observed in 22q11.2DS patients. To circumvent these limitations investigators have generated mouse models that replicate the symptoms observed in 22q11.2DS by removing a synthetic region in mouse chromosome 16 (Devaraju & Zakharenko, 2017) yet these mouse models, although valuable, have been used to a limited extend to identify cellular and molecular mechanism affected by the collective effect of all the genes mutated in the 22q11.2 segment.

Other approaches have included individually knocking down genes within the deletion regions; however, no single gene knockdown accounts for all the 22q11.2DS phenotypes. While this method has not revealed genes critical for all the physical and cognitive phenotypes of the syndrome, it has provided some understanding and attributing certain physical phenotypes. For example, *TBX1* knock-out mice have shown that heterozygous embryos have a high incidence aortic arch abnormalities – a typical cardiovascular phenotype seen in 22q11.2DS patients (Jerome & Papaioannou, 2001; Lindsay *et al.*, 2001).

Because of the large variety of genes affected in 22q11.2DS, pathways involving the mitochondria have been vastly overlooked. Past focuses of those studying 22q11.2DS often involved looking at possible neuronal pathways and studies of the more physical phenotypes of the syndrome such as craniofacial and cardiovascular abnormalities. Our lab decided to use a nonbiased approach to 22q11.2DS with the use of genealogical proteomics – an experimental paradigm created by our lab to uncover novel pathogenesis mechanisms and molecular phenotypes associated with polygenetic defects like 22q11.2DS (Zlatic *et al.*, 2018). Analysis of the 22q11.2 proteome showed an enrichment of mitochondrial proteins whose expression was sensitive to the 22q11.2 deletion. These finding shifted our focus to the relationship between 22q11.2DS and the mitochondria.

Preliminary Data and Experimental Approach

While little is known about which genes in the 22q11.2 segment contribute to 22q11.2DS's patient phenotypic traits and the cellular and molecular basis of these, our laboratory developed a human pedigree-based quantitative mass spectrometry strategy named *genealogical proteomics* to aid in addressing the complexity associated with 22q11.2DS complex polygenic nature and

identify cellular and molecular mechanisms downstream of the whole genetic defect rather than to study isolated genes within the 22q11.2 chromosomal segment. This paradigm allowed us to statically rank all proteomic phenotypes in an unbiased fashion (Zlatic *et al.*, 2018). Fibroblasts were taken from patients with both 22q11.2DS and diagnosed psychosis and their unaffected family members. Four families were used for this study. Fibroblasts were compared within families to minimize genetic background noise. The proteome of fibroblasts was quantified and compared those of unaffected family members and patients with 22q11.2DS and psychosis. We identified cellular and molecular pathways within the Ch22q11-sensitive proteome that comprehensively encompass the effect of this complex 22q11.2 deletion in cellular mechanisms. Analysis of the 22q11.2 proteome showed an enrichment of mitochondrial proteins whose expression was sensitive to the 22q11.2 deletion (Fig. 2). The significant enrichment of these mitochondrial hits in the 22q11 DS patient cells provides evidence that the mitochondria is a central affected organelle in 22q11.2DS (manuscript in preparation).

> The aforementioned findings have led to the following central hypothesis in our laboratory: defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome contribute to behavioral and synaptic phenotypes shared by schizophrenia and other neurodevelopmental disorders. This hypothesis and its foundations provide the framework for my honor thesis.

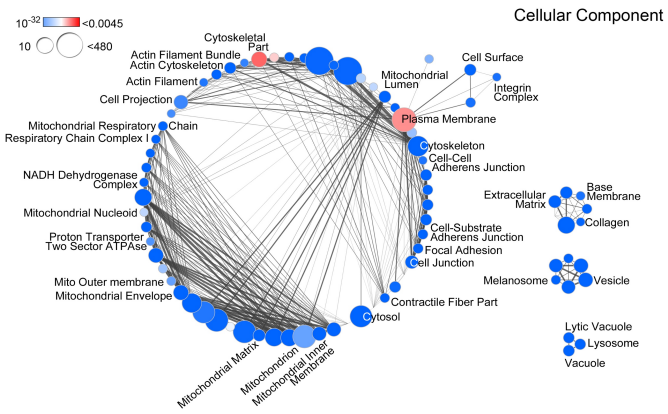

Figure 2. Interactome derived from genealogical proteomics study of four 22q11.2DS patients and unaffected family members. Size of circle represents the number of genes per ontological category. The color represents the p-value. The thickness of lines represents the number of genes shared per category.

Mitochondria and Chromosome 22q11.2 Deletion Syndrome

The chromosome 22q11.2 region contains six genes that have been identified to encode mitochondrial proteins: *Prodh, Slc25a1, Mrpl40, Zdhhc8*, *T10,* and *Txnrd2*. Our *genealogical proteomics* found that among the proteins affected in 22q11DS patients were components of the electron transport chain subunits and inner mitochondrial membrane transporters. Furthermore, immunoblots probing for the expression of mitochondrial transport chain subunits and inner mitochondrial transporters showed altered levels of SLC25A1 (mitochondrial citrate transporter), SLC25A4 (mitochondrial ADP/ATP carrier), and mitochondrial ribosome subunits in 22q11.2DS patients. While SLC25A1 is a part of the core microdeletion region in the 22q11.2 deletion, SLC25A4 and mitochondrial ribosome genes, with the exception of MRPL40, are not. These findings motivated the current study of SLC25A1 as a gene upstream of SLC25A4 and mitochondrial ribosome protein expression. Thus, we propose that SLC25 transporters family members, in particular SLC25A1, could modulate mitochondrial ribosome-dependent protein synthesis activity.

The SLC25A1 gene is a member of the mitochondrial carrier system. The mitochondrial carrier system regulates the function of the mitochondria and the cytoplasm by transporting small molecules between the two compartments. Among the mitochondrial carrier system is the SLC25A family – a 53-member group of canonical transporters (Taylor, 2017). The role of SLC25A1 within the mitochondria is to exchange citrate and malate across the inner mitochondrial membrane (Taylor, 2017). Little research has been done targeting SLC25A1 specifically, and its contribution to Chromosome 22q11.2 Deletion Syndrome is mostly unexplored.

Mitochondria and Schizophrenia

The notion that the mitochondrial pathways may play a mechanistic role in the pathogenesis of schizophrenia is not a novel idea in the field of schizophrenia research. It has been shown that abnormalities in the white matter – including increased glucose demand signaling a depletion of oxygen – in the frontal cortex of schizophrenia may be linked with an excess of reactive oxygen species (ROS). The Electron Transport Chain (ETC) is linked to the availability of glucose, oxygen, and ROS generation (Prabakaran *et al.*, 2004). Multiple studies have shown alterations in Complex I – IV of the ETC. These changes in expression levels are observed in various brain regions – particularly reduced activity of complex I, III, and IV in the temporal cortex and complex IV in the frontal cortex. These changes are not restricted to brain tissue as blood cells with downregulations of the ETC subunits are also observed in in schizophrenia patients, both at the transcript and protein levels (Ben-Shachar, 2002; Blass, 2002; Prabakaran *et al.*, 2004; Chauhan *et al.*, 2012). This compromised brain energy metabolism, oxidative stress, and downregulation of ETC subunits provide evidence of mitochondrial dysfunction in the prefrontal cortex of schizophrenia patients. Mitochondrial dysfunction can result in abnormal cellular energy

states which affects neurodevelopment – providing further evidence that the mitochondria may play a key role in the neurodevelopmental aspect of schizophrenia (Rajasekaran *et al.*, 2015). Other studies, including *in vivo* imaging and postmortem studies, have also shown energy metabolism impairment in the brains of schizophrenia patients (Maurer *et al.*, 2001; Scaglia, 2010). Abnormal mitochondrial morphology, size and density have also been seen in brains of schizophrenia patients (Cavelier *et al.*, 1995). These studies support the findings from our *genealogical proteomics* study regarding the role of the mitochondria in the pathogenesis of schizophrenia. Our study and the previous literature I have discussed provide evidence for the importance exploring the relationship between the mitochondria and 22q11.2DS.

Hypothesis and Aim

Based on the preliminary data discussed above, my hypothesis is that ***SLC25A1 null cells possess altered mitochondrial protein synthesis leaving them more susceptible to mitochondrial ribosome protein synthesis inhibiting drugs.***

Aim: Analyze the effects of various agents that selectively block mitochondrial proteins synthesis on mitochondrial respiration rates of SLC25A1 null cells.

From this, I predict that SLC25A1 null cells should have differential susceptibility to agents that selectively block mitochondrial proteins synthesis as compared to wild type cells. The focus of this project is to better understand how selectively targeting the mitochondrial ribosome effects protein synthesis in SLC25A1 null cells – a gene located in the 22q11.2 region – and to better understand the role of SLC25A1 in 22q11.2DS. The role of SLC25A1 within the mitochondria is to exchange citrate and malate across the inner mitochondrial membrane (Taylor, 2017). Little research has been done targeting SLC25A1 specifically, and its contribution to

Chromosome 22q11.2 Deletion Syndrome is mostly unexplored. SLC25A1 has not been previously associated with mitochondrial protein synthesis.

To gain more knowledge about the importance of SLC25A1 and its downstream mechanisms in the 22q11.2DS cells, I will focus on mitochondrial proteins synthesis. I will challenge cells with small molecule agents that selectively target mitochondrial ribosomes. Tetracycline and its derivatives have been known to perturb mitochondrial ribosome function and inhibit mitochondrial translation (Skrtic *et al.*, 2011; Chatzispyrou *et al.*, 2015). In addition, we chose several antibiotics targeting bacterial ribosomes with the ability to co-target mitochondrial ribosomes but not the mammalian ribosomes. A variety of drugs targeting the small and large subunits of the mitochondrial ribosome were tested, including: doxycycline, minocycline, chloramphenicol, and linezolid. Doxycycline and minocycline are tetracycline derivatives that target the 30S subunit of the ribosome and blocks the attachment of tRNA to the ribosome. Chloramphenicol targets the 50S subunit of the ribosome and inhibits peptide bond formation. Linezolid binds to the A site of the 50S subunit and prevents the formation of the initiation complex (Fig. 3) (Wilson, 2014). Actinonin inhibits peptide deformylase – an enzyme that catalyzed the removal of a formyl group from the starter methionine as the polypeptide chain exits the ribosomal exit tunnel (Fig. 4). Inhibition of peptide deformylase leads to stalled mitochondrial ribosome, which in turn triggers a ribosome and RNA decay pathway (Chen *et al.*, 2000; Richter *et al.*, 2013). Finally, carbenicillin is a beta lactamase inhibitor that does not show effect on mitochondrial ribosomes which was used as a control (Drawz & Bonomo, 2010).

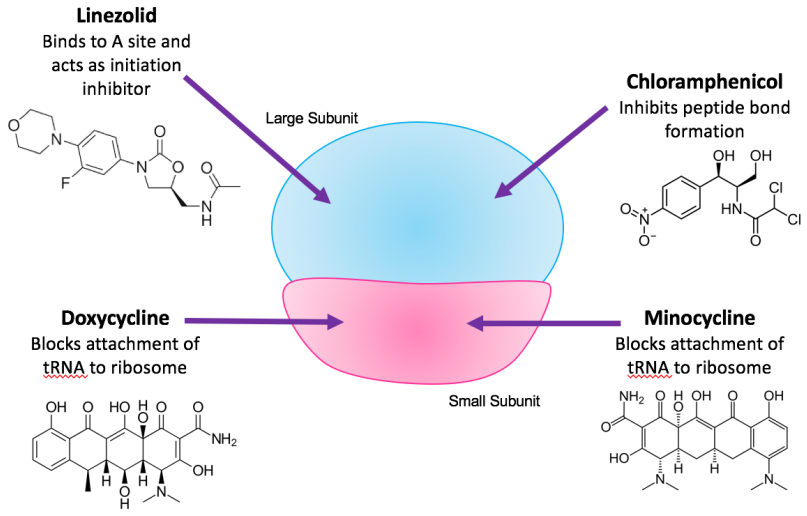

Figure 3. Doxycycline, linezolid, and chloramphenicol possess differing structural chemistry and hinder the mitochondrial ribosome through varying mechanisms of action. Minocycline and doxycycline are both tetracycline derivatives and share a mechanism of action.

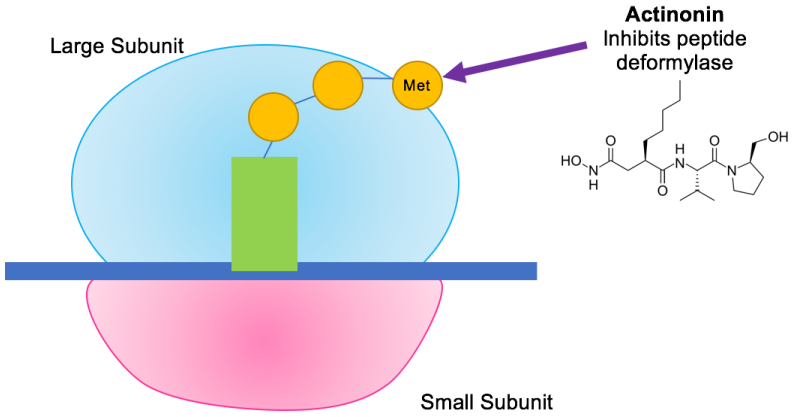

Figure 4. Actinonin inhibits peptide deformylase which prevents the removal of a formyl group from the starting methionine of a polypeptide chain. The presence of the formyl group prevents the peptide chain from properly exiting the mitochondrial ribosome.

To analyze the effect of these antibiotics on mitochondrial protein synthesis of SLC25A1 null cells, I will use Agilent Seahorse XF96 Extracellular Flux Analyzer to run a Mitochondrial

Stress Test on SLC25A1 knockout HAP1 cells treated with the above defined antibiotics. The Seahorse Assay allows for the measurement of oxygen consumption rate (OCR) as an indicator for mitochondrial respiration levels. The Seahorse Assay is robust and highly reproducible with low inter and intra-assay variations (Wang *et al.*, 2015). Previous analysis done by our lab has shown that untreated SLC25A1 KO HAP1 cells have a lower mitochondrial respiration rate than control HAP1 cells, providing support for findings in the *genealogical proteomics* that showed hits on the ETC and supports the notion that the mitochondria is a central organelle affected in 22q11.2DS. Using the Seahorse Assay, we can test the susceptibility of SLC25A1 KO HAP1 cells to agents that target ribosomal mitochondrial function compared to control HAP1 cells.

Mitochondrial Respiration and Protein Synthesis

If the focus is to study the effects of a deletion is SLC25A1 on protein synthesis, why are we studying mitochondrial respiration? The Electron Transport Chain, also known as the Mitochondrial Respiratory Chain, generates adenosine triphosphate (ATP) for the cell (Fig. 5). ATP is responsible for providing 90% of the energy needed for physiological activities within the cell. Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) control mitochondrial function. Mitochondrial DNA contains 37 genes which code for 13 polypeptides necessary for Complexes I, III, IV, and V of the ETC to function properly (Chauhan *et al.*, 2012). These necessary polypeptides are produced by the mitochondrial ribosome. By inhibiting the mitochondrial ribosome with targeting antibiotics, assembly of these ETC complexes are inhibited, thus leading to mitochondrial dysfunction and decreased mitochondrial respiration levels. We predict that cells lacking SLC25A1 will be more susceptible to mitochondrial ribosome targeting agents than control cells.

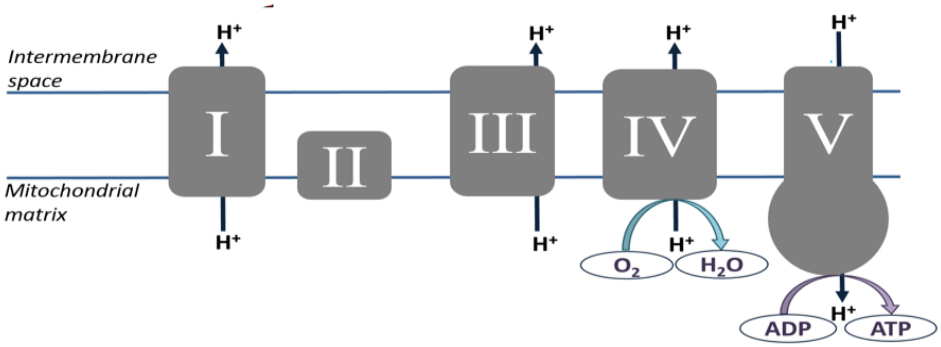

Figure 5. The Electron Transport Chain and its individual subunits (adapted and modified from Agilent Seahorse Microplates).

CHAPTER 2: MATERIALS AND METHODS

Tissue Culture

The cell lines used for this project were all HAP1 cells – a near-haploid human cell line derived from the male chronic myelogenous leukemia cell line (Kotecki *et al.*, 1999; Carette *et al.*, 2009). Three cell lines were used: HAP1 control cells, HAP1 SLC25A1 knockout SLC25A1$^{\Delta}$, and HAP1 SLC25A1 knockout SLC25A1$^{\Delta 1}$. The HAP1 SLC25A1 knockout cell lines were created by Horizon Discovery Group using CRISPR-Cas9. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum. The cells were incubated in a sterile 5% carbon-dioxide injected chamber at 37ºC. Cells were lifted, split, and passaged following standard tissue culture protocol as follows. Media was aspirated from the plate, washed twice with phosphate buffered saline (PBS) and incubated at room temperature with a 1:10 dilution of Trypsin in PBS. The reaction was stopped by adding 1ml IMDM media and mechanical force of a Pipette Aid was used to lift cells from the plate. The cell suspension was then transferred to a 15ml conical and centrifuged for 5 minutes at 800 rmp at 4ºC. Liquid was aspirated away from the cell pellet, and the pellet was suspended in PBS, or complete media further supplemented with antibiotic reagents. Depending on the size of the pellet, cells were seeded to passage plates at 1:10 – 1:20 onto new 10 cm plates. The cells were then placed in the incubator until the next passage.

Antibiotic Reagents

Drugs used were: doxycycline (Sigma-Aldrich, catalog # D3447) linezolid (Sigma-Aldrich, catalog # PZ0014), chloramphenicol (Sigma-Aldrich, catalog # C0378), actinonin (Sigma-Aldrich, catalog # A6671), and carbenicillin (Sigma-Aldrich, catalog # C1389). Doxycycline and linezolid were dissolved in DMSO. Chloramphenicol and actinonin were dissolved in ethanol. Carbenicillin was dissolved in filtered Milli-Q water. Two days prior to the

assay, optimal drug concentrations were added to the cells and the cells were incubated for 24 hours (Table 1).

Antibiotic	**Optimal Antibiotic Concentrations**
Doxycycline	2.25 uM
Chloramphenicol	2.5 uM
Linezolid	2.5 uM
Actinonin	5 uM
Carbenicillin	2.5 uM

Table 1. Optimal concentrations of antibiotics used to pretreat cells 48 hours before the Seahorse Mitochondrial Stress Test assays were run.

Seahorse Assay

To measure mitochondrial respiration levels, a Mitochondrial Stress Test was run using Agilent Seahorse Assay (Brand & Nicholls, 2011; Divakaruni *et al.*, 2014).

One day prior to assay

Cells were lifted using trypsinization following standard tissue culture protocol as mentioned previously. Cells were suspended in complete IMDM media and counted using the BioRad Cell Counter and Trypan blue. Cell concentrations were adjusted to the optimal target concentration (7.5×10^5 cells/ml). Optimal cell count per well using the Seahorse XFe Analyzer for HAP1 cells is 60,000 cells/well. At 7.5×10^5 cells/ml, 80 ul of suspension will result in 60,000 cells/ml. During plating, the pipette tip is held at a 45º angle to the side of the well. Cell suspension is allowed to rest for 30min while cells adhere to the plate. Complete media was added to each

well to a total volume of 180ul. A minimum of 1 row was left without cells but containing complete media for background readings.

The sensor cartridge was hydrated in Seahorse Calibrant at 37ºC in a non-CO_2 injected incubator overnight. Additionally, assay media was prepared by combining Seahorse Base Medium, 10mM D-glucose, 1mM Sodium Pyruvate, and 2mM L-glutamine. The media was stored at 37ºC until ready for use.

Day of the assay

Seahorse assay media was brought to pH of 7.4 with 0.1 M of sodium hydroxide. The assay media was then stored in the non-CO_2 injected incubator for at least 20 minutes before use. The wells of the Seahorse plate were washed two times with 170 ul complete Seahorse assay media. After aspirating off the final wash Seahorse Assay Media, wells were brought to a final volume of 180 ul with complete Seahorse assay media. The Seahorse plate was placed in non-CO_2 injected incubator at 37ºC for at least 20 minutes.

The 10x stress test drug dilutions were prepared in complete Seahorse assay media using: 20uM Oligomycin, 1.25uM FCCP, and 5uM Rotenone/Antimycin A. These were further diluted during assay injections to final concentrations of 2uM, 0.125uM, and 0.5uM respectively. The stress test drugs were placed into ports A, B and C on the probe tray, respectively.

The Seahorse Wave software recommendations were followed using the parameters obtained from the manufacture's protocol. Oxygen consumption was measured 12 times with a 3-minute mix period prior to each measurement. Ports were injected after every 3 measurements. Wave software was used to review and analyze results.

In order to normalize the data, a Thermofisher BCA protein assay was used on each Seahorse Assay plate. After the Seahorse assay was complete, the Seahorse plate was immediately placed on ice and washed 2 times using PBS supplemented with 100uM calcium chloride and 1mM magnesium chloride. The PBS/Ca^{2+}/Mg^{2+} was aspirated off, leaving approximately 10uL of cell solution in each well. Twenty microliters of a lysis buffer solution was then added to each well. The plate was placed on a rocker at -4ºC for 30 minutes. The plate was placed back on ice and varying concentrations of 2ug/mL Bovine Serum Albumin (BSA) were added to the blank wells. Reagent A (22ml) and Reagent B (440ul) from the BCA assay kit were mixed, and 200uL of the solution was added to each well using mechanical force to mix the solution. The plate was then placed in the non-CO_2 injected incubator for 30 minutes. After incubation, the plate was then placed in the Biotek microplate reader and protein concentration was read using Gen5 software. Protein concentration levels were then imported into Wave software to normalize the data from the Seahorse assays. At least three Seahorse assays with the same antibiotic treatments were combined for statistical analysis using the Seahorse Multi-File XF Cell Mito Stress Test Report Generator.

CHAPTER 3: RESULTS

My goal was to better understand how mitochondrial protein synthesis, and in-turn mitochondrial function, is affected by the removal of the SLC25A1 gene. Mitochondrial ribosome function was tested using Agilent Seahorse Assay Mitochondrial Stress Test which measures oxygen consumption rates (Divakaruni *et al.*, 2014). During the Seahorse Assay, cells were injected with four compounds over the course of 110 minutes (Fig. 6). When the cells were first placed in the Seahorse machine, a basal respiration level was measured for 20 minutes. After the basal respiration rate of the cells were taken, Oligomycin was injected. Oligomycin inhibits ATP synthase (Complex V of the electron transport chain) and allows the respiration rate associated to ATP production to be measured. After 30 minutes of Oligomycin treatment, FCCP was injected. FCCP uncouples the hydrogen ion gradient across the inner mitochondrial membrane, which limits oxygen consumption in the electron transport chain. By uncoupling the hydrogen ion gradient, the cells capacity for maximal oxygen consumption can be measured. Lastly, Rotenone and Antimycin A were simultaneously injected to inhibit Complex I and Complex III of the electron transport chain (Divakaruni *et al.*, 2014). These compounds completely block the mitochondrial electron transport chain and allow non-mitochondrial respiration levels to be measured (Fig. 6).

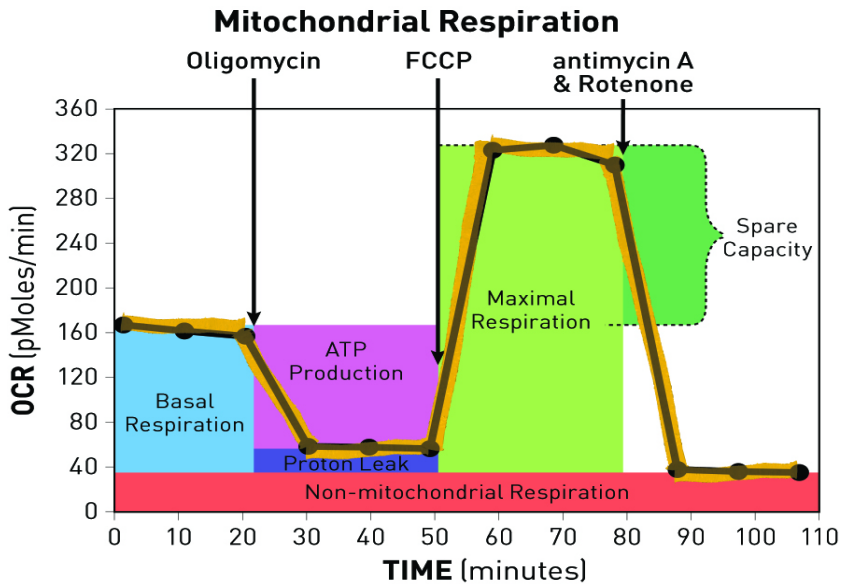

Figure 6. Schematic of an Agilent Seahorse Mitochondrial Stress Test measuring oxygen consumption rates (taken and modified from Agilent Seahorse Microplates).

SLC25A1 KO HAP1 cells show deficits in mitochondrial respiration

The oxygen consumption rate of two SLC25A1 KO HAP1 clone cell lines (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) were compared to the oxygen consumption rate of control HAP1 cells. As shown in Figure 2, the basal respiration and maximal respiration levels of SLC25A1 KO HAP1 cells are significantly decreased when compared to control HAP1 cells. In addition, ATP production of SLC25A1 KO cells is also decreased compared to control HAP1 cells. These results indicate that the absence of the SLC25A1 gene lead to deficits in mitochondrial respiration compared to wildtype cells (Fig. 7).

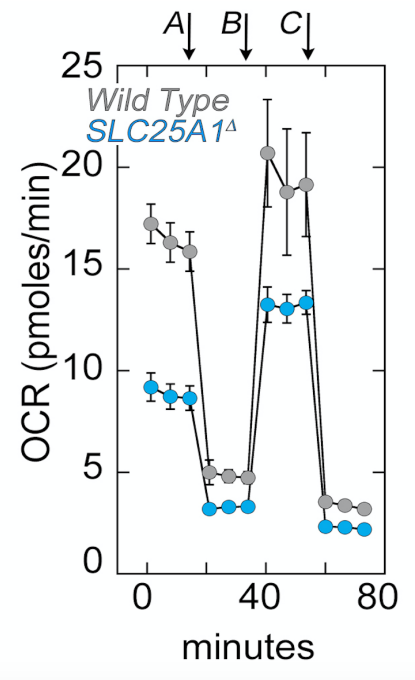

Figure 7. Time curve from a Seahorse Mitochondrial Stress Test illustrating the significant decrease in oxygen consumption rates between SLC25A1 KO HAP1 cells and control HAP1 cells. “A” represents oligomycin injection. “B” represents FCCP injection. “C” represents rotenone and antimycin A injection. Average ± SD

SLC25A1 KO HAP1 cells have a time dependent response to the antibiotic, doxycycline

SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) and HAP1 control cells were treated with 2.25uM doxycycline for various time periods ranging from 0 to 5 days. Shown in Figure 3, HAP1 control cells did not show changes in oxygen consumption rates when treated with various time exposures of doxycycline. In contrast, SLC25A1 KO HAP1 cells showed a time dependent response to doxycycline, where oxygen consumption rates decreased as the time exposed to doxycycline increased (Fig. 8).

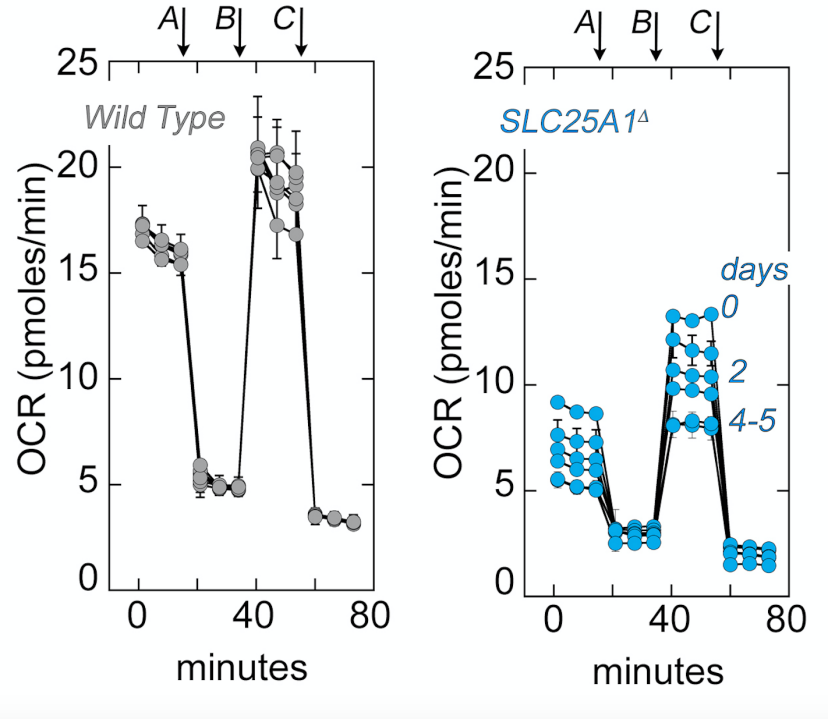

Figure 8. HAP1 control cells do not show a time dependent response to 2.25uM doxycycline. SLC25A1 HAP1 KO cells show a time dependent response to 2.25uM doxycycline. Average ± SD

SLC25A1 KO HAP1 cells treated with drugs targeting mitochondrial ribosomes show deficits in basal respiration rates

SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) and control HAP1 cells were treated with various agents targeting mitochondrial ribosome function and proteostasis. The cells were treated with: minocycline, doxycycline, chloramphenicol, linezolid, and actinonin. Seahorse Assays with minocycline were previously done by Dr. Stephanie Zlatic. Minocycline (1uM) treated SLC25A1 KO HAP1 cells showed a significant decrease in basal respiration levels compared to untreated SLC25A1 KO HAP1 cells (Figure 9a). Control HAP1 cells showed no change in basal respiration levels with treatment up to 1uM minocycline (Figure 9a). To ensure that this deficit in oxygen consumption rate was due to inhibition of mitochondrial protein synthesis and not spurious effects of these small molecules, I tested other agents that possessed different structural chemistries and mechanisms of action, yet which still targeted the mitochondrial ribosome (Fig. 14). No significant drop in basal respiration rates were seen HAP1 control cells when treated with the optimal concentration of any of the antibiotics used. As seen in Figure 9 and Figure 10, a significant reduction in basal respiration was seen in both clones of SLC25A1 KO HAP1 cells when treated with the optimal concentrations of the antibiotics: doxycycline (2.25uM: 6.34 pmol/min/ug protein to 4.08 pmol/min/ug protein, p=0.0448, 0.0022), chloramphenicol (2.5uM: 3.75 pmol/min/ug protein to 1.99 pmol/min/ug protein, p= 0.038, 0.0018), linezolid (2.5uM: 3.95 pmol/min/ug protein to 2.43 pmol/min/ug protein, p= 0.0016, 0.038), and actinonin (5uM: 4.14 pmol/min/ug protein to 2.33 pmol/min/ug protein, p=0.011, 0.0025). These results demonstrate that SLC25A1 KO HAP1 cells have a higher susceptibility to antibiotics targeting mitochondrial ribosome function than Control HAP1 cells.

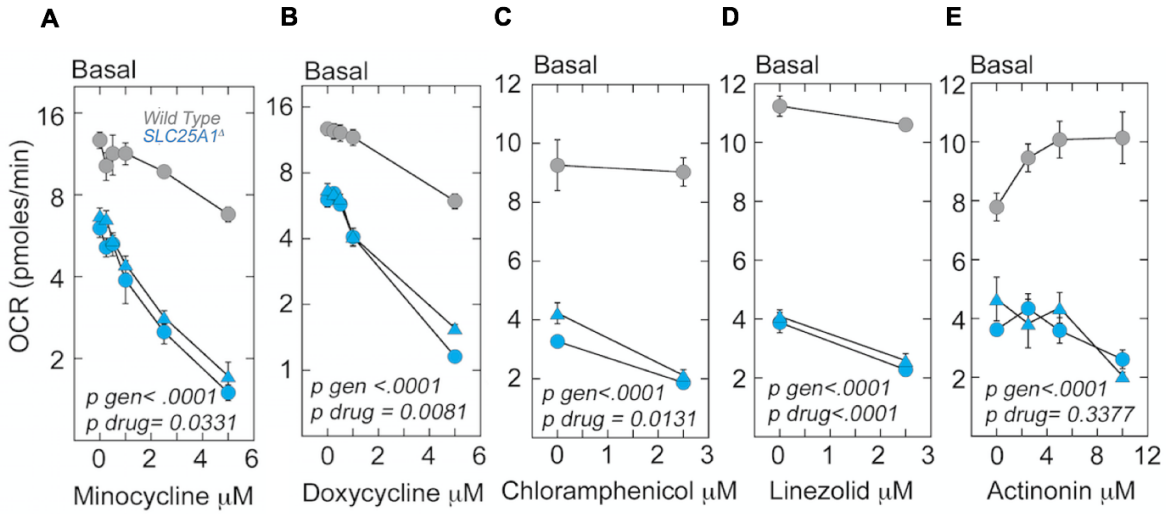

Figure 9. Concentration curves of basal respiration levels of SLC25A1 KO HAP1 cells and control HAP1 cells. HAP1 control cells are represented in grey. SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) are represented in blue. P values were calculated with Two-Factor ANOVA with Repeated Measures using Vassar Stats http://vassarstats.net/. Average ± SE

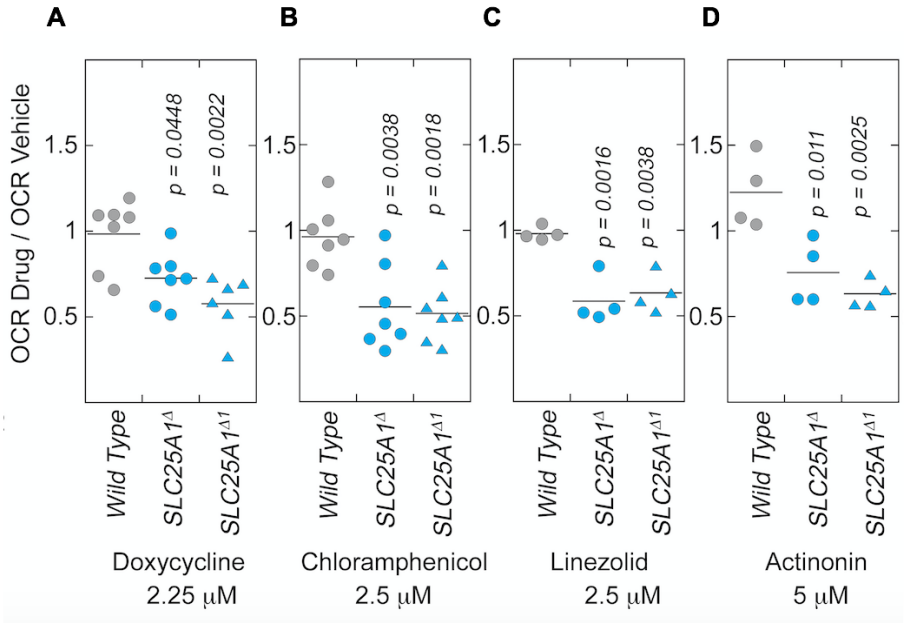

Figure 10. Fold of change graphs representing changes in basal respiration rates when cells were untreated compared to treatment with the optimal antibiotic concentration. Grey circles represent HAP1 control cells. Blue circles and blue triangles represent SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$). Both clones of SLC25A1 KO HAP1 cells showed a significant reduction in basal respiration when treated with doxycycline, chloramphenicol, linezolid, or actinonin. Wild type cells showed no significant change in respiration rates. P values were

SLC25A1 KO HAP1 cells treated with drugs targeting mitochondrial ribosomes show deficits in ATP-dependent respiration

After basal respiration levels were measured in the Seahorse Assay, oligomycin was injected (Fig. 1). Oligomycin inhibits ATP synthase, commonly known as Complex V of the Electron Transport Chain (Brand & Nicholls, 2011). When ATP synthase is inhibited, ATP production decreases and ATP-dependent mitochondrial respiration levels can be measured. No decrease in ATP-dependent oxygen consumption rates was seen HAP1 control cells when treated with the optimal concentration of any of the antibiotics used. A reduction in ATP-dependent oxygen consumption was seen in both SLC25A1 KO HAP1 clone cells lines when treated with the optimal concentrations of the antibiotics: minocycline (1uM: 5.37 pmol/min/ug protein to 3.11 pmol/min/ug protein, Fig. 11a), doxycycline (2.25uM: 5.37 pmol/min/ug protein to 3.37 pmol/min/ug protein, Fig. 11b), chloramphenicol (2.5uM: 3.00 pmol/min/ug protein to 1.26 pmol/min/ug protein), linezolid (2.5uM: 2.37 pmol/min/ug protein to 1.78 pmol/min/ug protein), and actinonin (5uM: 3.35 pmol/min/ug protein to 1.77 pmol/min/ug protein).

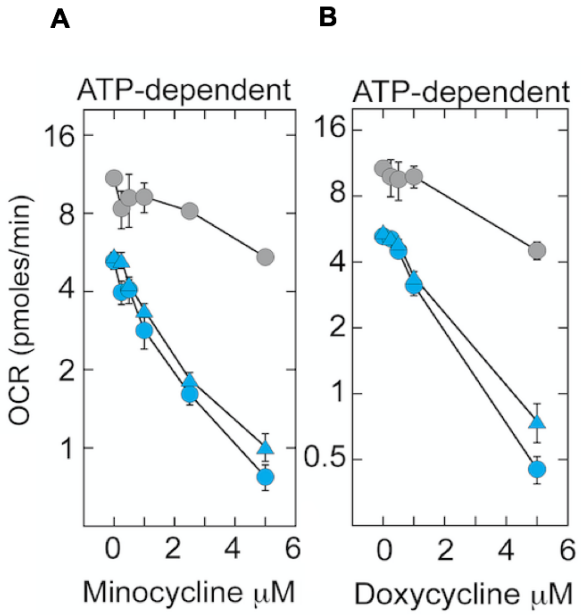

Figure 11. ATP-dependent mitochondrial respiration levels decrease in SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) as concentrations of minocycline and doxycycline increase. Dr. Stephanie Zlatic contributed to the minocycline and doxycycline datasets. Average ± SE

SLC25A1 KO HAP1 cells treated with drugs targeting mitochondrial ribosomes show deficits in maximal respiration rates

In order to measure maximal respiration levels, FCCP is injected during the Seahorse assay (Fig. 6). FCCP leads to an increase in respiration levels by uncoupling oxygen consumption from ATP production (Divakaruni *et al.*, 2014). No decrease in maximal respiration rates were seen HAP1 control cells when treated with the optimal concentration of any of the antibiotics used. A reduction in maximal respiration was seen in both clones of SLC25A1 KO HAP1 cells when treated with the optimal concentrations of the antibiotics: minocycline (1uM: 9.18 pmol/min/ug protein to 7.61 pmol/min/ug protein, Fig. 12a), doxycycline (2.25uM: 9.18 pmol/min/ug protein to 7.09 pmol/min/ug protein, Fig. 12b), chloramphenicol (2.5uM: 6.36 pmol/min/ug protein to 4.10 pmol/min/ug protein), linezolid (2.5uM: 5.95 pmol/min/ug protein to 4.63 pmol/min/ug protein), and actinonin (5uM: 5.06 pmol/min/ug protein to 4.26 pmol/min/ug protein).

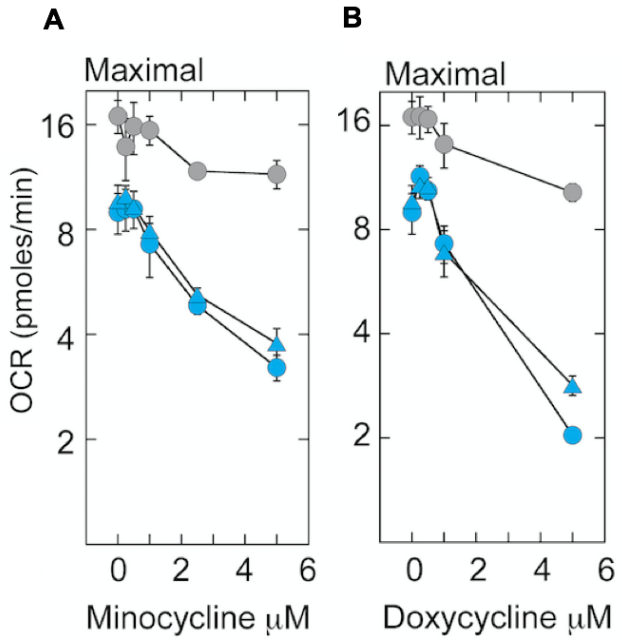

Figure 12. Maximal respiration levels for SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) as minocycline and doxycycline concentrations increase. Dr. Stephanie Zlatic contributed to the minocycline and doxycycline datasets. Average ± SE

SLC25A1 KO HAP1 cells show no change in respiration levels when treated with carbenicillin

Cells were treated with carbenicillin to ensure that the decrease in mitochondrial respiration levels of SLC25A1 KO HAP1 cells treated with antibiotics targeting the mitochondrial ribosome were due to mitochondrial dysfunction. Carbenicillin is a beta lactamase inhibitor (Drawz & Bonomo, 2010). Carbenicillin acts as a control since it should have no effect on the mitochondrial ribosome – thus having no effect on mitochondrial respiration levels. When treated with the optimal dosage of 2.5uM carbenicillin, SLC25A1 KO HAP1 cells (SLC25A1$^{\Delta}$ and SLC25A1$^{\Delta 1}$) showed no significant difference in basal respiration levels compared to untreated SLC25A1 KO HAP1 cells (Fig. 13). This result provides evidence that the significant decrease in respiration rates of SLC25A1 KO cells treated with antibiotics targeting

mitochondrial ribosomes is due to altered mitochondrial ribosome function and not to off-target effects of antibiotics in general. These results also provide evidence that SLC25A1 KO cells have increased susceptibility to agents targeting the mitochondrial ribosome.

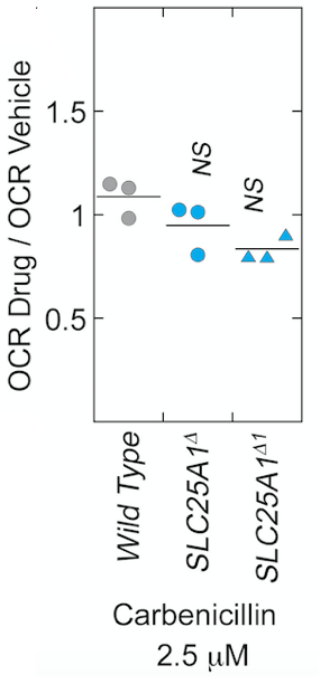

Figure 13. Fold of change graph indicating that there is no significant decrease in basal respiration levels between untreated SLC25A1 KO HAP1 cells and SLC25A1 KO HAP1 cells treated with 2.5uM carbenicllin. Grey circles represent HAP1 control cells. Blue circles and blue triangles represent SLC25A1 KO HAP1 cells ($SLC25A1^{\Delta}$ and $SLC25A1^{\Delta 1}$).

Summary of Results

My results show that SLC25A1 null cells possess decreased mitochondrial respiration levels as compared to control cells. Furthermore, SLC25A1 null cells have increased sensitivity to mitochondrial protein synthesis inhibiting drugs, regardless of structural chemistry or mechanism of action. This was further supported by a control assay with carbenicillin that showed no significant decrease in mitochondrial respiration levels in SLC25A1 null cells when treated with carbenicillin.

In summary, my results demonstrate that SLC25A1 null cells have increased susceptibility to mitochondrial ribosome protein synthesis inhibiting drugs strongly suggesting that SLC25A1 null cells possess impaired mitochondrial protein synthesis. My findings address my central hypothesis, which predicted SLC25A1 null cells have differential susceptibility to agents that selectively block mitochondrial proteins synthesis as compared to wild type cells.

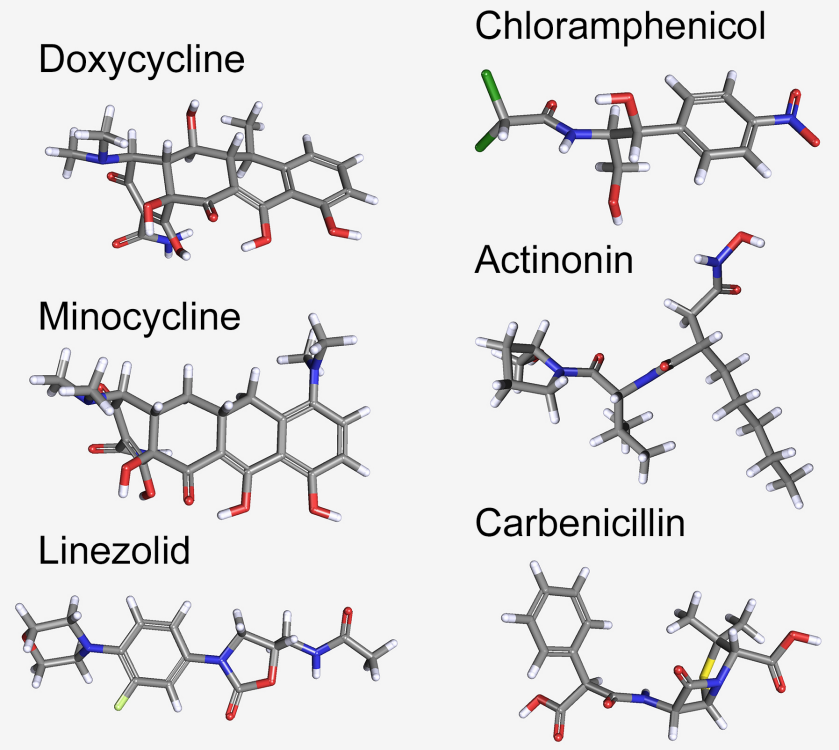

Figure 14. The 3-D structures of the antibiotics used in this study. Doxycycline, linezolid, and chloramphenicol possess differing structural chemistry and target the mitochondrial ribosome. While doxycycline and minocycline are both tetracycline derivatives, they possess some differing functional groups and configurations. Carbenicillin is a beta lactamase inhibitor, which does not target the mitochondrial ribosome. Structures generated with https://pubchem.ncbi.nlm.nih.gov/

CHAPTER 4: DISCUSSION

Summary of Results

The goal of this project was to investigate the interactions between mitochondrial transporters and mitochondrial protein synthesis in 22q11.2 Deletion Syndrome. Specifically, I focused on how mitochondrial protein synthesis is altered through the absence of the SLC25A1 gene – a gene which encodes the mitochondrial citrate transporter – and how this deficit leads to an increased susceptibility to mitochondrial ribosome protein synthesis inhibiting agents. This differential susceptibility was measured through mitochondrial oxygen consumption rates.

Previous studies in our lab revealed that the deletion of SLC25A1 in HAP1 cells led to decreased oxygen consumption rates compared to HAP1 control cells (Fig. 7). The basal respiration and maximal respiration levels of SLC25A1 KO HAP1 cells showed a decrease compared to HAP1 control cells. In addition, SLC25A1 KO HAP1 cells showed a decrease in ATP-dependent mitochondrial respiration compared to HAP1 control cells. This decrease in mitochondrial respiration in only the absence of the SLC25A1 gene indicates that the SLC25A1 gene is required for proper mitochondrial respiration.

To investigate the role of SLC25A1 in protein synthesis, I analyzed changes in oxygen consumption rates of SLC25A1 null cells when treated with drugs that inhibited mitochondrial protein synthesis compared to wildtype cells using Seahorse Mitochondrial Stress Test. This assay allowed me to measure and compare basal respiration rates, ATP-dependent mitochondrial respiration, and maximal respiration rates for SLC25A1 KO HAP1 cells and HAP1 control cells.

The Seahorse assays revealed that when SLC25A1 KO HAP1 cells were treated with a drug targeting mitochondrial protein synthesis – regardless of structural chemistry or mechanism of action – a decrease in oxygen consumption rates were seen in basal, ATP-dependent, and maximal respiration (Fig. 9, 10, 11, and 12). By using drugs that targeted multiple parts of the

mitochondrial ribosome, we were able to enhance our confidence that the drug effect was on the ribosome itself, and not off-target effects on the cell overall (Fig. 3 and 4). The drugs tested included: minocycline, doxycycline, chloramphenicol, linezolid, and actinonin. HAP1 control cells did not show these deficits in mitochondrial respiration levels when treated with the optimal concentration of any of these antibiotics (Fig. 9 and 10). These findings strongly suggest that SLC25A1 plays a role in mitochondrial protein synthesis, as the absence of SLC25A1 leads to an increased susceptibility to mitochondrial protein synthesis inhibiting drugs.

To ensure that the decreased mitochondrial respiration, and thus increased susceptibility to mitochondrial protein synthesis inhibiting drugs was actually due to mitochondrial dysfunction, Seahorse Mitochondrial Stress Test assays were done using carbencillin. Since carbencillin is a beta lactamase inhibitor, it had no effect on mitochondrial respiration levels in SLC25A1 KO HAP1 cells (Drawz & Bonomo, 2010) (Fig. 13). From this, we can conclude that the decrease in mitochondrial respiration levels in SLC25A1 KO HAP1 cells are due to deficits in mitochondrial protein synthesis caused by the lack of the SLC25A1 gene.

Future Directions

Quantitative proteomic analysis of SLC25A1

The results of this work suggest that mitochondrial protein synthesis is impaired when SLC25A1 is absent, but how SLC25A1 is linked to the impaired mitochondrial ribosome is still unknown. I propose that the study of the proteins that interact with SLC25A1 would provide clues as to the molecular mechanisms that could explain why the absence of SLC25A1 increases the susceptibility to mitochondrial protein synthesis inhibitors. The results of the SLC25A1 interactome may identify proteins that give further insight to the details regarding the linkage

between SLC25A1 and impaired mitochondrial ribosome function. In fact, preliminary results from our lab indicate that mitochondrial ribosome protein subunits and aminoacyl-tRNA ligases associate with SLC25A1 endogenously. These findings suggest a pathway to connect SLC25A1 with the increased susceptibility to mitochondrial protein synthesis inhibitors that I observed. A second approach that could address the mechanism by which SLC25A1 deficiency increases the susceptibility to mitochondrial protein synthesis inhibitors would be to perform quantitative *proteomics* on SLC25A1 null cells treated with the drugs from this study (minocycline, doxycycline, chloramphenicol, linezolid, and actinonin) to gather information on what proteins are upregulated and downregulated when treated with mitochondrial protein synthesis inhibiting drugs to see how these proteins may influence mitochondrial function. The Seahorse Mitochondrial Stress Test assays with drugs done for this project could then be repeated with knockout cells of proteins found in the interactome that may link SLC25A1 with mitochondrial ribosome function.

Mitochondrial respiration analysis of neuronal cells

This current study was done using HAP1 cells – a near-haploid human cell line derived from the male chronic myelogenous leukemia cell line. While using HAP1 cells provided many benefits, these cells may have differing metabolic profiles as neuronal cells. Repeating the current study using neuronal cells is of special interest, as 22q11.2DS has many cognitive phenotypes. As most neuronal ATP is generated through oxidative metabolism, neurons depend on mitochondrial function and oxygen supply and are very sensitive to mitochondrial dysfunction (Kann & Kovacs, 2007). In addition to differing metabolic profiles, neuronal cells would allow us to measure mitochondrial respiration rates in diploid cells lacking SLC25A1. Repeating this study in neuronal SLC25A1 KO cells would provide more insight and more support for my conclusion that the

removal of SLC25A1 leads to deficits in mitochondrial protein synthesis and that the absence of SLC25A1 leads to an increased subspecialty to mitochondrial protein synthesis inhibiting agents. In this regard, our laboratory is engineering a conditional mouse model that lacks SLC25A1. This tool will allow us to precisely and definitively test the hypothesis that mitochondrial protein synthesis machinery and SLC25A1 interact to specify mitochondrial function in neurons and synapses.

Induced pluripotent stem cells (iPSCs) from the fibroblasts of 22q11.2DS patients and family members have been developed by Dr. Avanti Gokhale in our lab. These cells were engineered by reprogramming somatic cells from fibroblasts into iPSCs (Roberts & Vetter, 2018). The iPSCs developed by Dr. Gokhale can be induced into a neuronal phenotype. If we were to repeat the current study in these cells, we would gain insight into how the hemizygous deletion affects mitochondrial respiration and protein synthesis as opposed to a complete knockdown of SLC25A1 seen in HAP1 cells. In addition, the iPSCs contain additional genes in the hemizygous deletion that may enhance or attenuate the phenotype of increased susceptibility to mitochondrial protein synthesis inhibiting agents.

Impact on understanding disease mechanisms

Schizophrenia's polygenic nature has created barriers to fully understanding the mechanisms of the disease. Researchers have done studies ranging from GWAS to exon sequencing and have yet to pinpoint causative disease mechanisms. This lack of understanding of the pathogenic mechanism of schizophrenia lead our lab to study 22q11.2DS. 22q11.2DS offered a well-characterized patient population at high genetic risk for developing schizophrenia. To address the complexity and polygenic nature of 22q11.2DS, our lab began our investigation into

this disease using *genealogical proteomics*, as this method allowed us to identify cellular and molecular mechanisms downstream of the genetic defect rather than individually isolating genes within the 22q11.2 deletion segment. When our *genealogical proteomic* study comparing 22q11.2DS patient fibroblast with unaffected family members revealed hits for mitochondrial-encoding genes, we uncovered a novel potential mechanism for 22q11.2DS. While it was known that the 22q11.2 deletion region contained 6 genes that coded mitochondrial proteins, 22q11.2DS research has tended to focus more towards exploring neuronal-specific pathways and genes relating to physical phenotypes such as cardiovascular and craniofacial malformations. Little research had been done to explore the role of the mitochondria in potential pathogenic mechanisms. Central to my project, the proteome showed hits on the Electron Transport Chain (ETC) and inner mitochondrial matrix. This lead to my interest in SLC25A1 – which encodes for mitochondrial citrate transporters and is located within the 22q11.2 deletion region – and how it affects mitochondrial protein synthesis. The idea that SLC25A1 is linked to mitochondrial protein synthesis is novel.

Through my work with SLC25A1 KO HAP1 cells, I discovered that SLC25A1 KO cells have decreased mitochondrial respiration levels, indicating that SLC25A1 plays an important role in mitochondrial protein synthesis. Through exposing SLC25A1 KO cells with drugs that inhibit mitochondrial protein synthesis through various mechanisms, I discovered that the absence of SLC25A1 leads to an increased susceptibility to deficits in mitochondrial protein synthesis. These discoveries are significant in taking steps towards better understanding the underlying biological mechanisms of both 22q11.2DS and schizophrenia.

The findings that antibiotics targeting the mitochondrial ribosome leads to significantly decreased respiration levels in SLC25A1 KO HAP1 cells is an important discovery, as antibiotics

like minocycline have been used to treat the negative and cognitive symptoms of schizophrenia (Solmi *et al.*, 2017). Although patients with 22q11.2DS only have a hemi-deletion of SLC25A1 – in comparison to the total absence of SLC25A1 in SLC25A1 KO HAP1 cells – their decreased expression of SLC25A1 still suggests that they may be at a genetic risk for increased mitochondrial ribosome susceptibility. As 22q11.2DS patients may take antibiotics targeting the mitochondrial ribosome, we must further explore the relationship between the mitochondria, neuropsychiatric conditions, and antipsychotic drugs.

In addition, further study needs to be done to better understand the linkage between SLC25A1 and mitochondrial ribosome dysfunction. Further studies into impaired mitochondrial pathways of neuropsychiatric disorders may unveil a better understanding of the pathogenic mechanisms contributing to psychiatric phenotypes. As more knowledge is gathered about the role of mitochondrial protein synthesis and the underlying pathways, we may discover new treatment options and new ways of early detection for neuropsychiatric disorders. The results of this project support the notion that impaired mitochondrial protein synthesis pathways play a part in the underlying pathogenic mechanism of neuropsychiatric disorders.

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