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# Dysfunctional Mitochondrial Biogenesis: A Potential Underlying Cause For Metabolic Diseases

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# DYSFUNCTIONAL MITOCHONDRIAL BIOGENESIS: A POTENTIAL UNDERLYING CAUSE FOR METABOLIC DISEASES

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## APPROVAL OF DISSERTATION

We, the faculty supervising the work of Caroline Ann Hunter, affirm that the dissertation, *Dysfunctional Mitochondrial Biogenesis: A Potential Underlying Cause for Metabolic Diseases*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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## DEDICATION

This dissertation is dedicated to my parents Kimbel and Julie Hunter, my siblings Kristie and Ryan Hunter, Justin Tomblin, and the rest of my family members. Your continued love and support have shown me that I can do anything. Without you, I would not be where I am today. I love you.

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## ABSTRACT

Mitochondria are essential organelles that play crucial roles in many aspects of cellular homeostasis. More importantly, the mitochondria are home to the majority of the metabolic pathways within the cell and are responsible for producing most of the cell's useable energy in the form of adenine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). In mammals, the majority of OXPHOS complex subunits are encoded by nuclear deoxyribonucleic acid (DNA); however, 13 core subunits essential for the function of OXPHOS complexes I, III, IV, and V are encoded in the mitochondrial (mt) DNA (mtDNA) and are synthesized within the mitochondria by its own transcription and translation machinery. Changes in the expression and post-translational modifications (PTMs) of OXPHOS subunits and mitochondrial proteins can be detrimental to mitochondrial energy production. In fact, alterations in mitochondrial functions impact cellular energy metabolism as well as influence whole-body metabolism and have been identified as underlying causes for several diseases including neurological disorders, insulin resistance, type 2 diabetes (T2D), and numerous cancer types. This has led to an extensive search for a better understanding of key players involved in mitochondrial function in disease states, in addition to new preventative and therapeutic strategies that are aimed at exploiting key components of mitochondrial biogenesis and energy metabolism. Our laboratory has shown that differences in mitochondrial biogenesis can be caused by changes in the sequence and/or PTMs of mitochondrial proteins, resulting in altered mitochondrial function and energy metabolism. In the present studies, we investigated changes in mitochondrial energy metabolism in two major metabolic diseases, T2D and liver cancer, and evaluated potential targets and therapies. We first investigated mitochondrial biogenesis and energy metabolism in T2D by studying the differences in the expression and activity of OXPHOS complexes in the liver and kidney of the polygenic

T2D model, TALLYHO/Jng (TH), and normal, C57BL/6J (B6), mice. A significant decrease was observed in the expression of both nuclear- and mitochondrial-encoded subunits of complexes I and IV, respectively, in TH mice compared to B6, which coincided with significant reductions in their enzymatic activities. Furthermore, we identified sequence variants in several mitochondrial proteins including OXPHOS complex subunits, a mitochondrial transfer ribonucleic acid (tRNA) synthetase, and mitochondrial ribosomal proteins (MRPs). The sequence variants identified in mitochondrial proteins might contribute to impaired mitochondrial biogenesis and energy metabolism by diminishing OXPHOS expression and activity in TH mice. In addition to T2D, we investigated liver cancer and demonstrated impaired mitochondrial OXPHOS complex expression and activity in cancerous liver biopsies compared to non-cancerous biopsies. The expression of the Src family kinases (SFKs), c-Src and Fyn, were increased in liver cancer cell lines and tissues. In fact, aberrant expression of c-Src kinase was observed in metastatic liver cancer tissues and the hepatic cell line Hep3B, which was correlated to a significant reduction in OXPHOS complex expression and activity. Additionally, increased c-Src expression was associated with decreased OXPHOS expression and activity in mouse embryonic fibroblast (MEF) cell lines, demonstrating the role of c-Src on mitochondrial OXPHOS in both health and disease. An inhibition of c-Src with the SFK inhibitor, PP2, and c-Src-specific small interfering ribonucleic acid (siRNA) alleviated the negative impact of c-Src on OXPHOS expression and improved mitochondrial energy metabolism while significantly impairing cell proliferation in normal and cancerous cells. In contrast, increased expression of Fyn kinase was associated with an elevated OXPHOS subunit expression in the liver cancer cell line, HepG2. Continuous OXPHOS activity can lead to high production of reactive oxygen species (ROS), which can cause mitochondrial damage; therefore, we explored the effects of Fyn

kinase, along with the effects of its inhibition on OXPHOS expression and cell proliferation, by treating cells with the SFK inhibitor SU6656 and natural antioxidants kaempferol and resveratrol. An increase in Fyn expression was associated with high OXPHOS expression, which was reduced in the presence of SU6656 and kaempferol. Furthermore, the treatment of the HepG2 cell line with SU6656, kaempferol, and resveratrol significantly inhibited cell proliferation. These treatments reduced OXPHOS activity and slowed cell growth by impairing cell proliferation in HepG2 cells, possibly by inhibiting Fyn activity. Evidence provided in these studies indicate that increased expression of c-Src and Fyn are found in liver cancer and more importantly regulate mitochondrial energy metabolism by altering the expression of OXPHOS complexes, which can contribute to the development of cancer and other metabolic diseases. Our data suggests that the suppression of SFKs can reduce cell proliferation and improve mitochondrial function and energy metabolism and should be further evaluated as targets for the treatment of liver cancer. Together, our studies demonstrated that changes in the expression of OXPHOS complexes due to sequence variants or PTMs, can significantly impair mitochondrial energy metabolism and may be underlying factors in both T2D and liver cancer. The identification of factors contributing to mitochondrial dysfunction will allow us to improve disease prognosis and treatments in various disease states.

# CHAPTER 1

# THE MITOCHONDRIA

## 1.1. The Mitochondria and its Genome

Mitochondria are one of the most complex, multifunctional organelles in the cell. The mitochondrion was derived from the engulfment of an alpha-proteobacterium by a precursor of the modern eukaryotic cell roughly two billion years ago (Gray, Burger, & Lang, 1999; Lane & Martin, 2010; D. Yang, Oyaizu, Oyaizu, Olsen, & Woese, 1985). The mitochondria are shaped by two membranes: 1) the outer membrane (OM) which is a smooth and continuous membrane that outlines the oblong shape of the organelle and forms an envelope to act as a barrier to restrict the passage of small molecules into the mitochondria and 2) the inner membrane (IM) which is highly invaginated (cristae) to increase its surface area to surround the inter soluble portion of the mitochondria, the mitochondrial matrix (MM), and is also the site of oxidative phosphorylation (OXPHOS) which is responsible for cellular respiration. The portion between the OM and the IM is the intermembrane space (IMS).

Over time, the mitochondria have been transformed to contribute to a variety of functions within the eukaryotic cell and play important roles in cell proliferation, senescence, apoptosis, immune response, detoxifying oxygen, ketone body production, heme biosynthesis, reactive oxygen species (ROS) production, and calcium and iron homeostasis (Ames, Atamna, & Killilea, 2005; Duchen, 2004; Osellame, Blacker, & Duchen, 2012). Although these pathways are vital to cellular function, perhaps the most significant function of the mitochondria is that they are the metabolic control center of the cell and contain the majority of metabolic pathways within the mitochondrial matrix, such as the citric acid cycle (CAC) or tricarboxylic acid cycle (TCA), fatty acid oxidation, and the urea cycle, all of which contribute to OXPHOS and energy production in

the cell. The primary role of the mitochondria is to convert the metabolites derived from the catabolism of proteins, carbohydrates, and fats to carbon dioxide ($CO_2$) and water ($H_2O$), using OXPHOS to drive adenosine triphosphate (ATP) synthesis.

During mitochondrial evolution, most genetic information was transferred to the nucleus, leaving the mitochondria with its own genome containing relatively few genes. In mammals, the mitochondrial (mt) deoxyribonucleic acid (DNA) (mtDNA) contains less than 1% of the total cellular DNA and has multiple copies inside the matrix of a single mitochondrion. The mitochondrial genome is a double-stranded (heavy- (H-) and light- (L-) strands), circular molecule that is roughly 16,569 base pairs in length and contains 37 genes (Anderson et al., 1981; Bibb, Van Etten, Wright, Walberg, & Clayton, 1981; Gray, 2012; Taanman, 1999). The structure of the mtDNA, as well as the gene organization, is highly conserved among mammals and is compact with nearly a complete absence of non-coding regions (Taanman, 1999; Wolstenholme, 1992). The mitochondrial genome encodes two ribosomal ribonucleic acids (RNA) (rRNAs), 22 transfer RNAs (tRNAs), and 11 messenger RNAs (mRNAs), two of which are bi-cistronic (Fig. 1). Additionally, the mtDNA also encodes the displacement loop (D-loop) which is a triple-stranded structure that encompasses key promoters for transcription, contains the leading strand origin of replication, and is the major control site for mtDNA expression (Kasamatsu & Vinograd, 1974; Taanman, 1999; Walberg & Clayton, 1981).

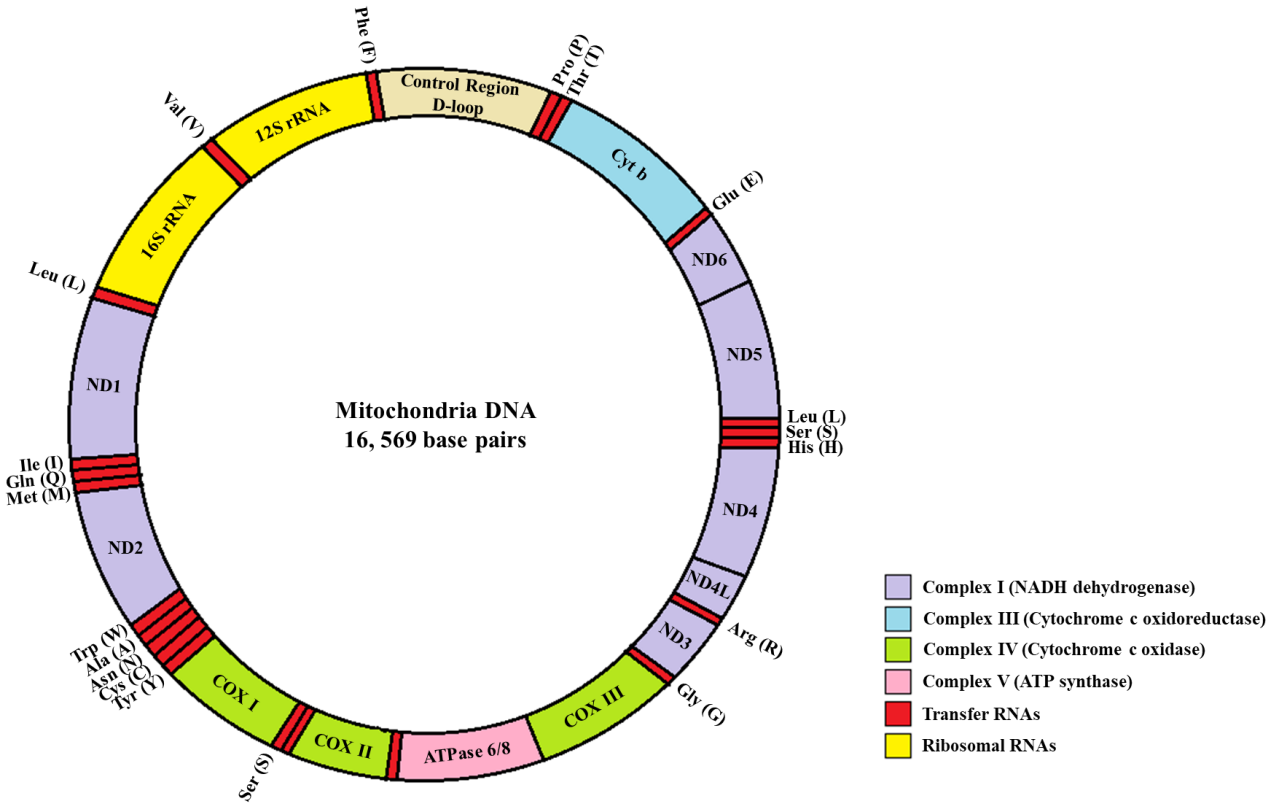


**Figure 1. The organization of the human mitochondrial genome.** The genome encodes 2 rRNA, 22 tRNA, and 13 mRNAs.

The mitochondrial genome encodes 22 tRNAs that are responsible for the synthesis of mitochondrial-encoded proteins. The mitochondrial tRNAs have a basic cloverleaf structure of canonical tRNAs but also have several distinguishing features that make them shorter and lack a number of conserved tertiary interactions (Helm et al., 2000; Wakita et al., 1994; Yokogawa et al., 1991). The compliment of mitochondrial tRNAs is sufficient to decode the simplified mitochondrial genetic code, which is distinct from the universal code, since each tRNA codes for one amino acid, except for leucine (Leu) and serine (Ser) which have two (Barrell, Bankier, & Drouin, 1979). In addition to its own tRNAs, the mitochondrial genome encodes two rRNAs, the 12S (small) and 16S (large), which are roughly only 25-30% of the total RNA (Christian & Spremulli, 2012; E. C. Koc & Koc, 2012; Pietromonaco, Denslow, & O'Brien, 1991).

The most important genes encoded in the mitochondrial genome are the 13 mRNAs which are essential proteins that compose the enzymatic cores of OXPHOS complexes I, III, IV, and V. Complex I (nicotinamide adenine dinucleotide plus hydrogen (NADH) dehydrogenase) is composed of seven mitochondrial-encoded proteins, NADH dehydrogenase (*ND*) *1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*; complex III (cytochrome (cyt) *c* reductase) contains one mitochondrial-encoded protein, *cyt b*; complex IV (cytochrome *c* oxidase (CO)) has three mitochondrial-encoded proteins, *COI*, *COII*, and *COIII*; and complex V (ATP synthase) contains two mitochondrial-encoded proteins, *ATP6* and *ATP8* (listed in Table 1). These 13 proteins are encoded by nine monocistronic and two dicistronic mRNAs which contain overlapping reading frames (Anderson et al., 1981; Anderson et al., 1982). For example, the first 46 nucleotides from the *ATP6* gene are the last nucleotides of the *ATP8* gene, while the last nucleotides on the *ATP6* gene are the first nucleotides of the *COIII* gene. These overlapping regions allow the mtDNA to remain densely packed.

| **Mitochondrial Complex** | **Mitochondrial Complex Number** | **Gene Name** |
|---|---|---|
| **NADH dehydrogenase** | I | *ND1* |
| | | *ND2* |
| | | *ND3* |
| | | *ND4* |
| | | *ND4L* |
| | | *ND5* |
| | | *ND6* |
| **Cytochrome *c* reductase** | III | *Cyt b* |
| **Cytochrome *c* oxidase** | IV | *COI* |
| | | *COII* |
| | | *COIII* |
| **ATP synthase** | V | *ATP6* |
| | | *ATP8* |

**Table 1. Mitochondrial-encoded OXPHOS subunits.**

Interestingly, the mitochondrial mRNAs are different than nuclear mRNAs because they lack a 5' untranslated region (5'-UTR) and have zero to eight nucleotides right before the start codon. Additionally, the start codons are located near the 5'-end of each mRNA and can be either AUG (adenine, uracil, guanine) or AUA (adenine, uracil, adenine) both of which encode methionine (Met), instead of the strict AUG in the nucleus and are used to start the transcription process of the mtDNA (Montoya, Ojala, & Attardi, 1981). Mitochondrial DNA replication, transcription, and translation are performed by their own machinery; however, besides the 37 genes (22 tRNAs, 2 rRNAs, and 13 mRNAs) encoded in the mtDNA, the remainder of the ~2,000 mitochondrial proteins are encoded in the nuclear DNA, synthesized on cytosolic ribosomes, and transported into the mitochondria.

### 1.2. Transcription and Translation in Mammalian Mitochondria

All mitochondrial functions are directly or indirectly linked to OXPHOS; therefore, mitochondrial gene expression is required for maintaining cellular homeostasis. The mitochondria utilize their own machinery to synthesize mitochondrial-encoded proteins, which is unique to mitochondria due to the two origins of the transcription and translation components. Most mitochondrial protein factors are encoded in the nuclear genome and all mitochondrial RNAs are encoded in the mitochondrial genome (D'Souza & Minczuk, 2018). Mitochondrial transcription begins in the major non-coding region of the genome at the L-strand (LSP) and H-strand (HSP) promoters (D'Souza & Minczuk, 2018; Shokolenko & Alexeyev, 2017). The LSP controls the transcription of eight tRNAs and the mitochondrial *ND6* gene, and the HSP, which is broken into 2 promoter systems HSP1 and HSP2, control the transcription of different mitochondrial genes. HSP1 produces transcripts for $tRNA^{Phe}$, $tRNA^{Val}$, and 2 ribosomal RNAs, and HSP2 spans the mitochondrial genome (Chang & Clayton, 1984; Montoya, Christianson,

Levens, Rabinowitz, & Attardi, 1982). Transcription factor A mitochondrial (TFAM) initiates transcription by binding to high-affinity sites upstream of the HSP1 and LSP promoters and induces a sharp bend in mtDNA, which may be important for the positioning of the remainder of the mitochondrial transcription machinery (Fisher, Lisowsky, Parisi, & Clayton, 1992; Ngo, Lovely, Phillips, & Chan, 2014). The complex formed between TFAM and mtDNA recruits the DNA-dependent RNA polymerase (POLRMT) and mitochondrial transcription factor B2 (TFB2M), which facilitate promoter recruitment of substrate to the POLRMT catalytic site (Morozov et al., 2014; Sologub, Litonin, Anikin, Mustaev, & Temiakov, 2009). TFB2M initiates the elongation step once it dissociates from POLRMT, recruiting the mitochondrial transcriptional elongation factor (TEFM) to the elongation complex (Minczuk et al., 2011; Sologub et al., 2009). TEFM further promotes POLRMT activity and stimulates the formation of longer transcripts (Posse, Shahzad, Falkenberg, Hallberg, & Gustafsson, 2015). Once the length of the polycistronic transcript has been processed, transcription termination is initiated and carried out by mitochondrial termination factor 1 (MTERF1) (M. Martin, Cho, Cesare, Griffith, & Attardi, 2005; Yakubovskaya, Mejia, Byrnes, Hambardjieva, & Garcia-Diaz, 2010). Mitochondrial transcription results in primary transcripts that need to be processed. The mRNAs undergo maturation and stabilization by cleavage and polyadenylation, and the tRNAs synthesized also undergo processing such as the addition of CCA tails and chemical nucleotide modifications (D'Souza & Minczuk, 2018; Shokolenko & Alexeyev, 2017). Additionally, tRNAs are aminoacylated and charged with a cognate amino acid, which is performed by mitochondrial aminoacyl tRNA synthetases (ARS2s), such as aspartyl-tRNA synthetase 2 (DARS2) (Chihara, Luginbuhl, & Luo, 2007). After processing of the mitochondrial mRNAs and tRNAs, they are assembled into the mitochondria ribosome for translation.

The mammalian mitochondria contain their own ribosomes that sediment as 55S particles and are made of 28S (small) and 39S (large) subunits (Christian & Spremulli, 2012; O'Brien, 1971). The mitochondrial ribosomes are associated with the inner membrane to allow the mitochondrial-encoded OXPHOS complex subunits to be synthesized near their sites of insertion (Vogel, Bornhovd, Neupert, & Reichert, 2006). Interestingly, mitochondrial ribosomes are more closely related to bacterial ribosomes than the eukaryotic counterparts, but mainly differ in composition and proteins. Mitochondrial ribosomes are slightly larger and more porous due to the higher protein to RNA ratio (69% to 31%) which is opposite of the bacterial ribosome composition (33% to 67%) (M. R. Sharma et al., 2003). The mitochondrial ribosomal large subunit (LSU) and small subunit (SSU) are composed of mitochondrial ribosomal proteins (MRPs), most of which have homologous ribosomal proteins in bacteria. The mitochondrial large subunit is involved in catalyzing the peptidyl-transferase reaction (Kaushal et al., 2014; E. H. Koc, ME; Spremulli, LL, 2010) and is comprised of 48 MRPs, 15 of which are mitochondrial specific, and the mitochondrial-encoded 16S rRNA (E. C. Koc, Burkhart, Blackburn, Moyer, et al., 2001; O'Brien et al., 2000; Suzuki et al., 2001b). The small subunit (SSU) provides the platform for the binding and decoding of the mitochondrial mRNAs (Kaushal et al., 2014; E. H. Koc, ME; Spremulli, LL, 2010) and is composed of 29 MRPs, 8 mitochondrial specific, and the mitochondrial-encoded 12S rRNA (E. C. Koc, Burkhart, Blackburn, Koc, et al., 2001; O'Brien et al., 2000; Suzuki et al., 2001a). Recently, all of the MRPs have been identified using mass spectrometry-based proteomic approaches (E. C. Koc, Burkhart, Blackburn, Koc, et al., 2001; E. C. Koc, Burkhart, Blackburn, Moyer, et al., 2001; Suzuki et al., 2001a, 2001b), but their functions are not yet fully understood. In addition to their involvement in mitochondrial translation, some MRPs have extra ribosomal functions and are involved in other cellular

regulatory processes. In fact, the mitochondrial ribosomal protein of the small subunit (MRPS) 29 (MRPS29) and 30 (MRPS30) are also involved in programmed cell death or apoptosis (Cavdar Koc, Burkhart, Blackburn, Moseley, & Spremulli, 2001; Cavdar Koc, Ranasinghe, et al., 2001; E. C. Koc, Burkhart, Blackburn, Koc, et al., 2001).

Although some of the details of the mitochondrial translation system remain to be discovered, the mammalian mitochondrial protein biosynthesis takes place in four phases, each of which require a set of auxiliary factors: 1) initiation, 2) elongation, 3) termination, and 4) recycling. Briefly, the initiation phase results in a completed 55S initiation complex with the help of mitochondrial initiation factors 2 and 3 (mtIF2 and mtIF3) (Christian & Spremulli, 2012; E. C. Koc & Spremulli, 2002; Schwartzbach, Farwell, Liao, & Spremulli, 1996; M. R. Sharma et al., 2003; Spurio et al., 2000). The 55S initiation complex moves into the elongation phase of protein synthesis, which is responsible for peptide elongation on the ribosome and involves key mitochondrial proteins such as the mitochondrial elongation factor Tu (mtEF-Tu), elongation factor G1 (mtEF-G1), and elongation factor Ts (mtEF-Ts) (Cai, Bullard, Thompson, & Spremulli, 2000a, 2000b; Christian & Spremulli, 2012; Schwartzbach et al., 1996; Schwartzbach & Spremulli, 1989, 1991). The elongation process adds one amino acid on at a time, until the stop codon appears in the A-site of the ribosome and is recognized by the mitochondrial translation release factor 1a (mtRF1a) which is released from the ribosome, along with the completed peptide. The ribosome complex is then dissociated and translation factors are recycled with the help of the mitochondrial ribosome recycling factors 1 and 2 (mtRRF1 and mtRRF2), so another round of protein synthesis can begin (Christian & Spremulli, 2012; Chrzanowska-Lightowlers, Pajak, & Lightowlers, 2011; Y. Zhang & Spremulli, 1998).

Mitochondrial translation takes place at the IM due to the location of the mitochondrial ribosomes. This also allows the 13 synthesized mitochondrial-encoded OXPHOS subunits to be inserted directly into their respective complexes, which is a co-translational process (Ott & Herrmann, 2010). The synthesis of the mitochondrial-encoded proteins by their own machinery is essential for OXPHOS function (Fig. 2). The OXPHOS complexes are contributions from both the nuclear and mitochondrial DNA, two physically and functionally separate genomes. Disruption of the synthesis of mitochondrial proteins on either genome could be detrimental to OXPHOS and mitochondrial energy metabolism. Therefore, the coordinated expression and regulation of both nuclear- and mitochondrial-encoded subunits is essential for the assembly and function of OXPHOS complexes to prevent impaired energy production and mitochondrial dysfunction, which can cause several disease states to emerge.

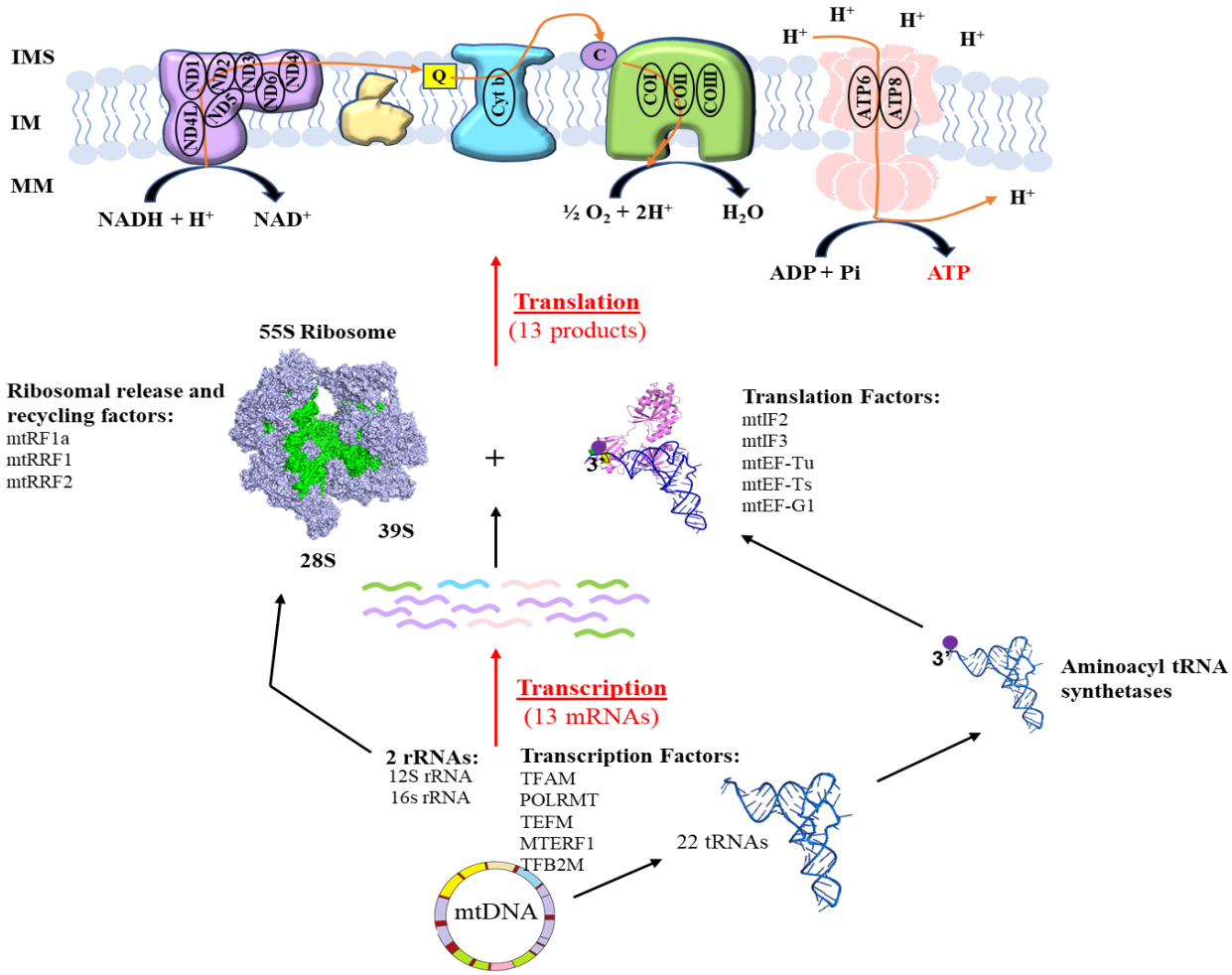


**Figure 2. Overview of mitochondrial transcription and translation.** Mammalian mitochondria contain a ~16.5 kilobase circular genome (mtDNA) that encodes two rRNAs, 22 tRNAs, and 13 mRNAs. The mtDNA is transcribed by its own transcription and translation machinery, which is responsible for the synthesis of the 13 mitochondrial-encoded subunits of complexes I (purple), III (blue), IV (green), and V (ATP synthase) (pink). The mtDNA is transcribed by its transcription machinery (TFAM, POLRMT, TEFM TFB2M, MTERF1, and ARS2s). The mammalian mitochondrial 55S ribosome (28S and 39S subunits) and rRNAs, along with other translation factors, such as mitochondrial initiation factors (mtIF2 and mtIF3), mitochondrial elongation factors (mtEF-Tu, mtEF-Ts, and mtEF-G1), and mitochondrial ribosomal release and recycling factors (mtRF1a, mtRRF1, and mtRRF2), are responsible for the synthesis of the 13 mitochondrial encoded subunits of complexes I (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*), III (*cyt b*), IV (*COI*, *COII*, and *COIII*), and V (*ATP6* and *ATP8*). The bovine 55S mammalian ribosome (PDB # 3J9M) was modeled and shown in light purple with mitochondrial rRNAs highlighted in green. Bovine mtEF-Tu (PDB #1XB2) and Escherichia coli aminoacyl $tRNA^{Cys}$ (PDB # IB23) were modeled to show the interaction of tRNA with mtEF-Tu and are presented in violet and blue, respectively. Figure adapted from (C. A. Hunter et al., 2019).

### 1.3. The Role of Mitochondria in Metabolic Disorders

The mitochondria host many pathways that are necessary for ATP production. Mitochondrial dysfunction, caused by pathogenic alterations and changes in the expression of mitochondrial proteins resulting in diminished ATP and increased ROS production, is an underlying factor in the development and progression of many metabolic diseases, including cancer, cardiovascular disease, obesity, and type 2 diabetes (T2D) (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Dhalla, Temsah, & Netticadan, 2000; Garcia-Escudero, Martin-Maestro, Perry, & Avila, 2013; Hassanein & Frederick, 2004; Kelley, He, Menshikova, & Ritov, 2002; J. A. Kim, Wei, & Sowers, 2008; E. C. Koc et al., 2015; Sayre, Smith, & Perry, 2001; Wallace, 1999). Mitochondrial dysfunction has been associated with characteristics ranging from poor mitochondrial quality to reduced mitochondrial number and size to the inability to properly undergo cellular respiration by OXPHOS and build new mitochondria, mitochondrial biogenesis, in numerous disease states. Therefore, a better understanding of the cause of mitochondrial dysfunction and the effects on cellular signaling in diseases is essential to determine if the functional impairments are remedial and to discover a potential for novel treatments to target these deficiencies.

Mitochondrial OXPHOS plays a central role in cellular metabolism and impaired OXPHOS function can be the cause of disease pathogenesis. While OXPHOS disorders can be caused by mutations in the nuclear or mitochondrial genomes, there remains a lack of understanding in the pathogenic mechanisms that result in clinical symptoms observed in many patients. Mutations in the mitochondrial OXPHOS genes encoded within the mitochondrial and nuclear genomes, along with reduced expression and/or changes in PTMs result in lower OXPHOS expression, activity, and reduced ATP generation in metabolic disorders (Chandra &

Singh, 2011; Minocherhomji, Tollefsbol, & Singh, 2012). Additionally, OXPHOS inhibition can result in the toxic accumulation of ROS, which can damage nucleic acids, lipids, and mitochondrial and cellular proteins, including the iron-sulfur centers of OXPHOS complexes I, II, and III, resulting in diminished mitochondrial energy production and impaired mitochondrial function (Wallace, 1999, 2005). Due to the complexity of the mitochondria in numerous critical cellular functions which rely on OXPHOS activity, identifying key factors involved in the deregulation of OXPHOS in metabolic diseases such as T2D and cancer is crucial.

### 1.3.1. Mitochondrial Function, OXPHOS, and Insulin Signaling

Mitochondria play critical roles in numerous cellular processes, especially energy production by OXPHOS since almost all cellular processes require ATP. Therefore, both physiological alterations in energy demand and fuel supply influence mitochondrial biogenesis, dynamics, and mitophagy (Liesa & Shirihai, 2013; Ruegsegger, Creo, Cortes, Dasari, & Nair, 2018). Since mitochondrial OXPHOS is a major function of the mitochondria and plays a pivotal role in normal cellular processes, its deregulation can be detrimental to cellular metabolism and has been suggested as a global pathogenic mechanism in the development of many diseases, especially insulin resistance and T2D (Smeitink, Zeviani, Turnbull, & Jacobs, 2006).

The prevalence of obesity and T2D is increasing rapidly and has recently grown to epic proportions worldwide due to an increase in sedentary lifestyles and increased consumption of calorie dense food. Although there are many factors that contribute to the development of obesity, insulin resistance, and T2D, mitochondrial dysfunction has been implicated in the development and progression of these disease states (Chow, From, & Seaquist, 2010; Kelley et al., 2002; J. A. Kim et al., 2008; Petersen et al., 2003; Rector et al., 2010).

Insulin resistance is a major characteristic in the pathogenesis of T2D and is the primary factor leading to insufficient pancreatic β-cell function. Insulin is the predominant hormone involved in fuel metabolism and is secreted in the pancreatic β-cell (Ashcroft & Rorsman, 2012; Muoio & Newgard, 2008) which is largely controlled by mitochondrial OXPHOS activity. Under normal conditions, glucose and other cellular fuels are oxidized to produce reducing equivalents that are fed into mitochondrial OXPHOS complexes and used to produce ATP. The increase in ATP closes the ATP-sensitive potassium ($K^+$) channel, depolarizes the plasma membrane of the β-cell, opens the voltage-gated calcium ($Ca^{2+}$) channels, and triggers the release of the cellular granule containing insulin (Fex et al., 2018; Henquin, 2000). This $K_{ATP}$-dependent pathway is dependent on mitochondrial OXPHOS activity, thereby demonstrating the essential role of the mitochondria in the control of insulin release in the pancreatic β-cell. Decreased insulin release has been associated with diminished mitochondrial function and reduced OXPHOS activity (J. A. Kim et al., 2008). This results in the inability of tissues to respond to physiological levels of insulin, which leads to insulin resistance and the development of T2D.

Interestingly, insulin secretion is controlled by mitochondrial function and OXPHOS activity; however, insulin is also one of the hormones that regulates mitochondrial functions and has been identified as a critical regulator of mitochondrial biogenesis in numerous tissue types (Ruegsegger et al., 2018). The presence of insulin stimulates the activities of the glucose transporter 4 (GLUT4) and fatty acid transport protein (FATP), enhancing the uptake of these substrates by the cell, which are then oxidized and fed into the mitochondrial OXPHOS for ATP production (Y. Zhang & Ye, 2012). Insulin has also been shown to promote mitochondrial oxidative energy metabolism and ATP synthesis by inducing pyruvate dehydrogenase (PDH) activity through dephosphorylation to enhance glucose utilization (Y. Zhang & Ye, 2012).

Insulin promotes mitochondrial function by increasing substrate availability for oxidation as well as controlling mitochondrial biogenesis and metabolism (Y. Zhang & Ye, 2012). Briefly, in the insulin signaling pathway, insulin binds to the insulin receptor and induces receptor autophosphorylation, causing a downstream signaling cascade that leads to the recruitment and phosphorylation of insulin receptor substrates 1 and 2 (IRS1 and IRS2) and activation of the AKT pathway (De Meyts, 2000; Ruegsegger et al., 2018). The insulin/IRS/AKT pathway plays an important regulatory role in mitochondrial function and oxidative metabolism. This pathway increases the expression of nuclear- and mitochondrial-encoded genes by inhibiting Forkhead box O1 (FOXO1) and activating the mammalian target of rapamycin (mTOR). The inhibition of FOXO1 influences the activity and expression of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) which is an inducible transcriptional coactivator that has been shown to interact with nuclear respiratory factors-1and 2 (NRF-1 and NRF-2) and stimulate the transcription of nuclear-encoded mitochondrial genes (Cheng et al., 2009; Heilbronn, Gan, Turner, Campbell, & Chisholm, 2007; McCarty, 2005; Mootha et al., 2003). PGC-1α also enhances mitochondrial gene transcription through transcription factor A, mitochondrial (TFAM). The activation of mTOR causes the downstream activation of other targets responsible for the increased synthesis of nuclear-encoded mitochondrial proteins involved in the TCA cycle, β-oxidation, and OXPHOS (Cunningham et al., 2007; Ruegsegger et al., 2018; Schieke et al., 2006). Collectively, these findings demonstrate insulin signaling is critical in the mitochondrial metabolic activity of the cell and is clearly involved in the synthesis and expression of OXPHOS complexes. Therefore, identifying key players in mitochondrial dysfunction in insulin resistance is essential to determine if mitochondrial dysfunction is a cause or consequence of insulin resistance and the pathogenesis of T2D.

#### 1.3.1.1. Mitochondrial Dysfunction in Obesity and Type 2 Diabetes

Defects in OXPHOS and energy production is one of the central factors contributing to T2D and insulin resistance. In fact, reduced gene and protein expression has been observed and reported in multiple subunits of each of the five OXPHOS complexes, leading to significant decreases in ATP synthesis in both animal models and human subjects with obesity, T2D, and insulin resistance (Haythorne et al., 2019; Heilbronn et al., 2007; Heinonen et al., 2015; Hou et al., 2017; Patti et al., 2003; Rong et al., 2007; Segerstolpe et al., 2016; Skov et al., 2007). Several studies also demonstrated reduced enzymatic activity along with decreased expression of OXPHOS complexes in obesity and/or T2D (Kelley et al., 2002; Ruegsegger et al., 2018; Simoneau, Veerkamp, Turcotte, & Kelley, 1999). Additionally, studies have also shown that the activity of mitochondrial enzymes that control OXPHOS activities, such as PDH, are also inhibited in diabetic and insulin resistant conditions in various tissues in animals and human subjects (Alves et al., 2011; Mondon, Jones, Azhar, Hollenbeck, & Reaven, 1992; Y. Zhang & Ye, 2012). These studies show significant impairment in ATP generation and fatty acid synthesis in most tissues, further demonstrating mitochondrial dysfunction by reduced energy metabolism. In an effort to uncover the role of mitochondrial dysfunction by OXPHOS in T2D and insulin resistance, many studies have investigated numerous mitochondrial proteins involved in mitochondrial biogenesis and energy metabolism.

Mitochondrial function is largely controlled by mitochondrial biogenesis and is determined by the size, number, and quality of the mitochondria (Fex et al., 2018). Interestingly, in the subcutaneous adipose tissue (SAT) and skeletal muscle of obese and T2D subjects, reduced mitochondrial number, size, mass, and activity was observed (Heinonen et al., 2015; Kelley et al., 2002). Abnormal cristae structure within the mitochondria in obese subjects has

also been reported (Bach et al., 2003; Joseph, Joanisse, Baillot, & Hood, 2012; Kelley et al., 2002), suggesting defects in the inner mitochondrial membrane, which could also alter mitochondrial function and OXPHOS activity.

Mitochondria are dynamic and undergo network remodeling by continuous fusion and fission to adapt to changes in cellular energy demands. Under normal conditions, mitochondrial fusion results in the formation of a mitochondrial network that allows the redistribution of proteins and mtDNA between multiple mitochondria to prevent the accumulation of mutated or damaged mtDNA in a cell (Bo, Zhang, & Ji, 2010; Joseph et al., 2012; Otera & Mihara, 2011). The inhibition of mitochondrial fusion is commonly associated with increased ROS generation and can impair the insulin signaling pathways, leading to insulin resistance and T2D (Joseph et al., 2012). In fact, decreased expression and loss of function of mitofusin 2 (Mfn2) and optic atrophy 1 (OPA1) was observed and reported in T2D and insulin resistant subject and was shown to alter the mitochondrial morphology and impair mitochondrial metabolism and oxygen consumption in skeletal muscle and a variety of other tissues and cell types (Bach et al., 2005; Bach et al., 2003; H. Chen, Chomyn, & Chan, 2005; Kelley et al., 2002; Pich et al., 2005). Collectively, these studies were shown to decrease mitochondrial energy metabolism, further demonstrating mitochondrial dysfunction in T2D, insulin resistance and obesity.

Decreases in the amount of mtDNA and mitochondrial content have been reported in obese twins, as well as obese and insulin resistant adults and offspring of parents with T2D (Heinonen et al., 2015; Mootha et al., 2003; Patti et al., 2003; Petersen, Dufour, Befroy, Garcia, & Shulman, 2004; Sreekumar, Halvatsiotis, Schimke, & Nair, 2002). These findings implicate changes in mitochondrial biogenesis as a contributing factor to the development of insulin resistance, obesity, and T2D. Additionally, changes in mitochondrial gene expression and protein

synthesis have also been observed and contribute to alterations in mitochondrial biogenesis. Mitochondrial biogenesis relies on the coordinated regulation of both nuclear and mitochondrial protein expression for energy metabolism via OXPHOS. Interestingly, one of the most common pathogenic variants linked to T2D was found in the mitochondrial genome. The variant at position 3243 in the mtDNA substitutes adenine (A) with guanine (G) (A3243G) in the gene that encodes the mitochondrial tRNA which recognizes Leu, *tRNA(Leu)* (Kami et al., 2012; Maassen et al., 2004; Scaglia & Northrop, 2006). This mutation decreased mitochondrial protein synthesis and caused a defect in the expression and function of OXPHOS complexes, diminished ATP production, and impaired the insulin secretion response (Maassen et al., 2004).

In mammals, mitochondrial biogenesis is regulated by PGC-1α (Heilbronn et al., 2007; McCarty, 2005; Mootha et al., 2003). A coordinated reduction in the mitochondrial transcription-related factors PGC-1α and PGC1-beta (PGC-1β), and their responsive gene NRF-1 was found in obesity and T2D subjects and obese subjects with a family history of diabetes (Heinonen et al., 2015; Mootha et al., 2003; Patti et al., 2003). Additionally, changes in the expression of mitochondrial translation factors has also been observed in subjects with obesity and T2D and shown to alter energy metabolism by changes in OXPHOS. In fact, haploinsufficiency and/or the heterogenous expression of pentatricopeptide repeat domain 1 (PTCD1) resulted in decreased protein synthesis and impaired RNA processing, which was shown to cause adult onset obesity (Perks et al., 2017).

Additionally, the altered expression and/or sequence variants of numerous mitochondrial ribosomal proteins also contributes to reduced mitochondrial energy metabolism via impaired mitochondrial protein synthesis and T2D (Bains et al., 2004; Heinonen et al., 2015; Rong et al., 2007). One of the essential translation factors, mtEF-Tu, was identified as the most connected

mitochondrial protein to the insulin signaling cascade and is strongly associated with human obesity and T2D (Mercader et al., 2012). In obese Zucker rats, exercise was suggested to increase mitochondrial metabolism and improve impaired mitochondrial function. Exercise resulted in higher mRNA expression of mitochondrial transcription factor A (TFAM), mtIF2, and Tu translation elongation factor, mitochondrial (TUFM), while the protein expression of mtEF-Tu was only increased in lean Zucker rats (Greene et al., 2014). The lack of increase in mtEF-Tu expression in obese rats further implies that decreased regulation of mitochondrial translation elongation is a possible candidate in impaired mitochondrial biogenesis in obesity and T2D, demonstrating the role of mitochondrial translation in disease development.

Although many studies highlight the role of mitochondrial dysfunction in the development and progression of obesity, insulin resistance, and T2D, there is still a major gap in the molecular pathogenesis. Therefore, further studies should be performed to identify impaired mitochondrial proteins involved in the pathogenesis of the diseases.

### 1.3.2. Mitochondrial Dysfunction in Cancer

In addition to T2D and other metabolic diseases, mitochondrial dysfunction has also been established as an underlying player in various cancer types. One of the hallmarks of cancer is the metabolic reprogramming of cancer cells which not only alters energy metabolism but also supports the bioenergetic demand of the cell, synthesis of macromolecules, and cellular survival (Hanahan & Weinberg, 2011; Pavlova & Thompson, 2016; Vyas, Zaganjor, & Haigis, 2016). Since mitochondria are the major metabolic hubs of the cells, they are responsible for cellular reprogramming through multiple mechanisms. Interestingly, mitochondrial energy production as well as mitochondrial mass, dynamics, redox homeostasis, cell death regulation, and metabolic signaling influence the initiation, growth, survival, and metastasis of cancer cells (Vyas et al.,

2016). Therefore, their flexibility allows for cell growth and survival in adverse environmental conditions and contributes to tumorigenesis (Vyas et al., 2016).

Mitochondrial OXPHOS is required for specific phases of tumor progression causing mitochondria to be essential for cancer cells (Iommarini, Ghelli, Gasparre, & Porcelli, 2017; Wallace, 2012). However, mitochondrial dysfunction has been established as an underlying factor for tumorigenesis and has been observed in aggressive tumors (Gogvadze, Zhivotovsky, & Orrenius, 2010; Seyfried, 2015; Simonnet et al., 2002). In fact, in 1927 Otto Warburg observed that cancer cells rely more on glycolysis than OXPHOS for ATP production even in the presence of oxygen, known as the Warburg effect or aerobic glycolysis (Warburg, 1956a, 1956b; Warburg, Wind, & Negelein, 1927). Although the Warburg effect is a prominent feature of many cancer cells, this discovery suggested that mitochondrial defects might be central to cancer cell biology. Mitochondrial dysfunction of cancer cells is a result of impaired respiration and energy production caused at the genetic level by sequence variants in the nuclear- and mitochondrial-encoded proteins, or at the protein level, by PTMs of mitochondrial proteins.

#### 1.3.2.1. Mutations in Mitochondrial Genes in Cancer

For the past several decades, sequence variants in mitochondrial genes have been associated with an increased risk of cancer; however, it wasn't until recently that mutations in the mtDNA were also reported and linked to the development and progression of cancer (Horton et al., 1996; van Gisbergen et al., 2015). Recent studies have demonstrated the importance of mtDNA in cancer cells by replacing the cancer cell mtDNA with normal and pathogenic mtDNA, resulting in changes in the phenotypes (Ishikawa et al., 2008; Petros et al., 2005; Shidara et al., 2005). Due to the proximity of the mtDNA to damaging ROS, which is produced as a byproduct of OXPHOS, mtDNA is more susceptible to mutations. In fact, over 190 tumor-

specific mtDNA variants have been reported in various cancer types (Brandon, Baldi, & Wallace, 2006). Many mtDNA variants found in cancer cells inhibit OXPHOS, increase ROS production, and promote tumor cell proliferation leading to invasion and metastasis (pathogenic or deleterious mutations). Other variants facilitate in the adaptation of tumor cells to the environmental conditions and bioenergetic demands of the cell, such as increased tolerance for high ROS toxicity and exposure to pro-apoptotic conditions, without altering OXPHOS (adaptive or beneficial mutations) (Ashton, McKenna, Kunz-Schughart, & Higgins, 2018; Brandon et al., 2006; Wallace, 2012). Therefore, the identification of variants in mtDNA and their effects on mitochondrial function, OXPHOS, and energy production is essential to determine the relevance of pathogenic mutations in cancer development.

The mitochondria contain their own transcription and translation machinery, most of which is encoded in the nuclear genome. Interestingly, sequence variants have been found in some of the mitochondrial proteins involved in transcription and translation of the mtDNA and result in changes in the expression of OXPHOS complexes. For example, heterozygous variants in TFAM were associated with mtDNA depletion, increased mtDNA damage and oxidative stress, loss of certain OXPHOS complexes, and significant mitochondrial dysfunction in colorectal cancer (J. Guo et al., 2011; Han et al., 2011; Larsson et al., 1998; Woo et al., 2012). Additionally, decreases in the expression of MRPs, including mitochondrial ribosomal protein of the large subunit 11 (MRPL11), were identified by our laboratory and shown to impair OXPHOS expression and activity in head and neck cancer (E. C. Koc et al., 2015). Sequence variants were also found in the mitochondrial-encoded tRNAs encoding for tyrosine (Tyr), tryptophan (Trp), and leucine (Leu) and shown to impair mitochondrial protein synthesis and reduce the expression

of OXPHOS complexes in liver tumors and breast cancer (Bianchi, Bianchi, & Bailliet, 1995; Taira, Yoshida, Kobayashi, Yaginuma, & Koike, 1983).

Additionally, variants in the mtDNA encoding for OXPHOS subunits have been reported in various cancer types. Sequence variants in the coding region of the mtDNA have been found in colon cancer, endometrial cancer, and cervical cancer (Chinnery, Samuels, Elson, & Turnbull, 2002; Copeland, Wachsman, Johnson, & Penta, 2002; V. W. Liu et al., 2003; Zhai, Chang, Zhang, Liu, & Wu, 2011). Outside of the control region variants, variants have been reported in the *ND1*, *ND3*, *ND4*, *ND5*, and *ND6* genes of OXPHOS complex I, which were associated with structural alterations, decreased complex I activity, and truncated mRNA in renal adenocarcinoma, osteosarcomas, and invasive breast cancer (Canter, Kallianpur, Parl, & Millikan, 2005; Horton et al., 1996; Iommarini et al., 2014). Some of these variants were correlated with decreased OXPHOS activity and expression, increased ROS generation, increased resistance to apoptosis, and promotion of tumor growth (Cruz-Bermudez et al., 2015; Ishikawa et al., 2008; L. K. Sharma et al., 2011; van Gisbergen et al., 2015). Sequence variants were also found in the mitochondrial-encoded *COI* gene of complex IV in prostate cancer and epithelial ovarian cancer (Pereira, Soares, Maximo, & Samuels, 2012; Petros et al., 2005). Interestingly, variants within the mtDNA have been shown to influence cancer progression and metastasis, and the aggressiveness of cancer is often correlated with the decreased OXPHOS activity (Dasgupta et al., 2012; Ishikawa et al., 2008; Larman et al., 2012; V. W. Liu et al., 2001; Petros et al., 2005; Polyak et al., 1998; Simonnet et al., 2002).

During the genetic mapping of oncogenes, sequence variants in the nuclear-encoded mitochondrial proteins were identified in some cancers. Variants were found in enzymes involved in metabolic pathways that converge onto OXPHOS, such as fumarate hydratase (FH)

and isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) in numerous cancer types (Frezza et al., 2011; Lehtonen et al., 2004; Paschka et al., 2010; Picaud et al., 2011; Yan et al., 2009). Mutations were also found in the nuclear-encoded OXPHOS subunits of complexes I and II and shown to impair mitochondrial function. Decreases in the expression of NADH:ubiquinone oxidoreductase core subunit V1 (NDUFV1), NADH:ubiquinone oxidoreductase subunit A13 (NDUFA13), and NADH:ubiquinone oxidoreductase core subunit S3 (NDUFS3) of complex I were associated with increased metastatic behavior and mesenchymal properties (Gaude & Frezza, 2014; Kulawiec et al., 2008; Pereira et al., 2012; Taylor et al., 2010), demonstrating the correlation between epithelial to mesenchymal transition (EMT) induction and OXPHOS impairment. Additionally, variants were found in three of the subunits of succinate dehydrogenase (SDH) (complex II), SDH subunit B (SDHB), SDH subunit C (SDHC), and SDH subunit D (SDHD), in multiple cancer types (Ishii et al., 2005; Janeway et al., 2011; H. J. Kim & Winge, 2013; Ricketts et al., 2008).

Along with mutations in both nuclear- and mitochondrial-encoded proteins, changes in the mitochondrial number and amount of mtDNA were also shown to contribute to the decreased expression of OXPHOS complexes in many cancers including breast cancer, lung cancer, human hepatocellular carcinoma, non-small cell lung cancer, and gastric cancer (Ashton et al., 2018; Pedersen, 1978; van Gisbergen et al., 2015; Vyas et al., 2016). Surprisingly, OXPHOS was upregulated in some cancers such as leukemias, lymphomas, melanomas, ovarian cancer, and head and neck cancers, due to increased mitochondrial number and mtDNA amount which were stimulated to compensate for metabolic defects caused by sequence variants and increased oxidative stress (Ashton et al., 2018; van Gisbergen et al., 2015; Yu, 2011). The mitochondrial diversity between different cancers and within the same cancer type explains why mitochondrial

proteins are defined as both tumor suppressors and promotors, stimulate and inhibit mitochondrial biogenesis, and/or increase and decrease OXPHOS and energy production. These variations signify that mitochondrial biology and tumorigenic signaling intersects at multiple levels; therefore, it is essential to identify oncogenic variants or other regulatory mechanisms of mitochondrial proteins to better understand mitochondrial energy metabolism in cancer.

#### 1.3.2.2. Post-Translational Modifications in Cancer

Post-translational modifications (PTMs) are one of the critical cellular regulatory mechanisms that affects numerous biological functions and have been associated with the pathogenesis of many diseases, especially cancer (B.S. Sharma et al., 2019; V. Singh et al., 2017; Stram & Payne, 2016). PTMs such as acetylation, phosphorylation, methylation, and ubiquitination have emerged as dynamic regulators of target proteins that play key roles in cellular processes including signal transduction, cell growth and division, and protein synthesis (Hofer & Wenz, 2014; Paik, Paik, & Kim, 2007; Park, Jo, Kim, Kim, & Ahn, 2015). Phosphorylation is the most widespread class of PTMs and one of the most common signaling mechanisms used in signal transduction (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Due to its role in the regulation of cellular processes such as cell growth, signaling, and apoptosis in the healthy condition, deregulation of the signaling cascade and alterations in other phosphorylated pathways result in various cancer types (Hofer & Wenz, 2014; Marmiroli, Fabbro, Miyata, Pierobon, & Ruzzene, 2015; V. Singh et al., 2017). In cancer, overactive protein kinases are often due to an overexpression of activated kinases and/or mutations in the kinase genes that hold the kinases in an activated conformation (Marmiroli et al., 2015), resulting in stimulated signal transduction and increased cancer cell growth or metastasis.

Although serine (Ser) and threonine (Thr) phosphorylation have been well studied and identified as the most prevalent forms of phosphorylation, tyrosine (Tyr) phosphorylation has recently emerged as a major cellular regulatory mechanism (Z.Chen et al., 2016; Du, Zhang, Hassan, Biswas, &Balaji, 2010; Hofer & Wenz, 2014; Roskoski, 2005; B.S. Sharma et al., 2019). Alterations in the pathways caused by deregulated kinases result in several cancer types. In fact, the greatest number of oncoproteins are encompassed within the Tyr kinase family including members of the receptor and non-receptor tyrosine kinases (V. Singh et al., 2017; Takeuchi & Ito, 2011). Tyrosine kinases play critical roles in the modulation of growth factor signaling (Arora & Scholar, 2005); therefore, activated forms of these kinases cause tumorigenesis by increasing cell proliferation and growth, inhibiting apoptosis, and inducing metastasis. One of the major groups of tyrosine kinases commonly deregulated in cancer is the Src family kinases (SFKs). The Src family non-receptor protein tyrosine kinases play vital roles in the signal transduction pathways and are capable of inducing malignant transformations in epithelial and non-epithelial cancer types such as colorectal, breast cancer, liver cancer, melanomas, head and neck cancer, lung cancer, brain cancer, pancreatic cancer, and blood cancers (Sen & Johnson, 2011; Summy & Gallick, 2003; Yeatman, 2004; J. Zhao et al., 2013; R. Zhao et al., 2015). SFKs play prominent roles in tumor invasion and progression, metastasis, angiogenesis, epithelial-to-mesenchymal transition, resistance to apoptosis, and cell proliferation and migration (Sen & Johnson, 2011; Summy & Gallick, 2003). The contribution of SFKs to tumor development and progression remain to be completely defined, although are further discussed in this dissertation.

Interestingly, along with cell signaling pathways, SFKs have been shown to regulate numerous mitochondrial pathways, especially those involved in energy metabolism (Hebert-Chatelain, 2013; Hebert-Chatelain et al., 2012; Miyazaki, Neff, Tanaka, Horne, & Baron, 2003;

Salvi, Brunati, & Toninello, 2005). In fact, only five SFKs have been located within the mitochondria (Miyazaki et al., 2003; Salvi et al., 2002; Salvi et al., 2005; Vahedi, S., Chueh, Chandran, & Yu, 2015). Two of these mitochondrial SFKs, c-Src and Fyn, are ubiquitously expressed in all cell types and have been shown to regulate mitochondrial functions (Hebert-Chatelain, 2013; Hebert-Chatelain et al., 2012; E. C. Koc, Miller-Lee, & Koc, 2016; Thomas, Soriano, & Imamoto, 1995); yet their roles in the regulation of mitochondrial energy metabolism and OXPHOS in cancer are largely unknown. Since mitochondrial dysfunction is one of the hallmarks of cancer and SFKs have been shown to contribute to cancer development by the induction of the Warburg effect, a better understanding of the role of SFKs in the regulation of mitochondrial energy metabolism in cancer is essential. The effect of SFKs, c-Src and Fyn, on mitochondrial energy metabolism in health and disease, along with potential treatment options to inhibit SFKs and improve mitochondrial function and slow liver cancer progression, are explored in this dissertation.

# CHAPTER 2

# THE REGULATION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION AND ENERGY METABOLISM BY SRC FAMILY KINASES

## 2.1. Mitochondrial Regulation by Tyrosine Phosphorylation

Mitochondria are the major metabolic hubs of the cell and are crucial for energy generation and dysfunction of any of these pathways can lead to oxidative stress, energy deprivation, and the development of various pathologies. Therefore, due to their essential role in the cell, mitochondrial functions need to be tightly regulated, making many of the auxiliary factors involved in these processes targets for regulatory interventions. In the last several decades, post-translational modifications have emerged as powerful regulators of mitochondrial processes (Hofer & Wenz, 2014; E. C. Koc & Koc, 2012). Although acetylation has been frequently studied and shown to play a critical role in the regulation of mitochondrial energy metabolism in mammals (Guan & Xiong, 2011; E. C. Koc & Koc, 2012; Lu, Scott, Webster, & Sack, 2009; Sack, 2011), phosphorylation was identified as one of the primary ways to regulate mitochondrial processes to rapidly adjust protein activity without permanently altering its structure (Hebert-Chatelain, 2013).

Recently, Tyr phosphorylation has emerged as a major regulator of cellular pathways and has become more crucial as a regulator of mitochondrial proteins (Hebert-Chatelain, 2013; Salvi et al., 2005). In fact, of the 90 Tyr kinases identified in the human genome by Manning et al., 25 were located within the mitochondria (Manning et al., 2002; Pagliarini & Dixon, 2006; Roskoski, 2004). Of these 25 mitochondrial Tyr kinases, several belong to one of the major family of non-receptor Tyr kinases, Src family kinases (SFKs) (Manning et al., 2002; Salvi et al., 2002). To date, several SFK members have been shown to phosphorylate mitochondrial proteins

and regulate mitochondrial function and bioenergetics within the cells (Hebert-Chatelain et al., 2012; E. C. Koc et al., 2016; Miyazaki et al., 2003; Salvi et al., 2002; Salvi, Morrice, Brunati, & Toninello, 2007; Vahedi, Chueh, Chandran, & Yu, 2015), establishing their regulatory role on mitochondrial energy metabolism.

### 2.2. Src Family Kinases

c-Src is a non-receptor SFK that has been under investigation for several decades and has been shown to play key roles in a number of different cellular pathways, including cell growth and division, cell motility, differentiation, morphology, and survival (G. S. Martin, 2001; Roskoski, 2004, 2005). In 1911, Peyton Rous studied the Rous Sarcoma virus (RSV) and identified a transforming viral protein responsible for the development of chicken tumors, v-Src, along with its cellular homologue, c-Src, which were both established as activated tyrosine kinases (Brown & Cooper, 1996; Collett & Erikson, 1978; Czernilofsky et al., 1980; Levinson, Oppermann, Levintow, Varmus, & Bishop, 1978; Rous, 1911; Stehelin, Varmus, Bishop, & Vogt, 1976). In fact, due to these findings, c-Src became the first molecularly defined and characterized proto-oncogene. c-Src was encoded in a physiological gene in normal cells which resulted in an investigation of its function and a search for other related proteins. This led to the discovery of 11 proteins that had similar amino acid sequence homology, structural features, and functions to c-Src which formed what is now known as the SFKs: Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, Lyn, Frk, Srm, and Brk (Brown & Cooper, 1996; Manning et al., 2002; Thomas & Brugge, 1997).

The SFKs can be divided into 3 groups of enzymes based on their sequence homology: group I includes c-Src, Yes, Fyn, and Fgr, group II is made of Blk, Lyn, Lck, and Blk, and the distantly related group III consists of Frk, Srm, and Brk. These kinases have been found in

multiple different cell types with a restricted pattern of expression; however, three of the kinases, c-Src, Yes, and Fyn, are expressed in all cell types (Brown & Cooper, 1996; Thomas & Brugge, 1997). SFKs regulate many cellular pathways and couple with distinct cellular targets to activate downstream signaling to promote cell survival (Thomas & Brugge, 1997). SFKs are controlled and activated by a diverse class of cellular receptor tyrosine kinases (RTKs), including G-protein coupled receptors, integrin receptors, steroid hormone receptors, epidermal growth factor receptor (EGFR), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and cytokine receptors (Fleming et al., 1997; W. Mao et al., 1997; Munshi, Groopman, Gill, & Ganju, 2000; Thomas & Brugge, 1997). Additionally, the activation of c-Src and SFKs can lead to the upregulation of secretory factors involved in metastasis, migration, proliferation, adhesion, and invasion. SFKs are key downstream transducers that initiate cell signaling to promote survival by stimulating cellular growth and proliferation and maintaining cellular functions. More importantly, five of the SFK members are translocated into the mitochondria and regulate cellular energy metabolism, as shown in Fig. 3.

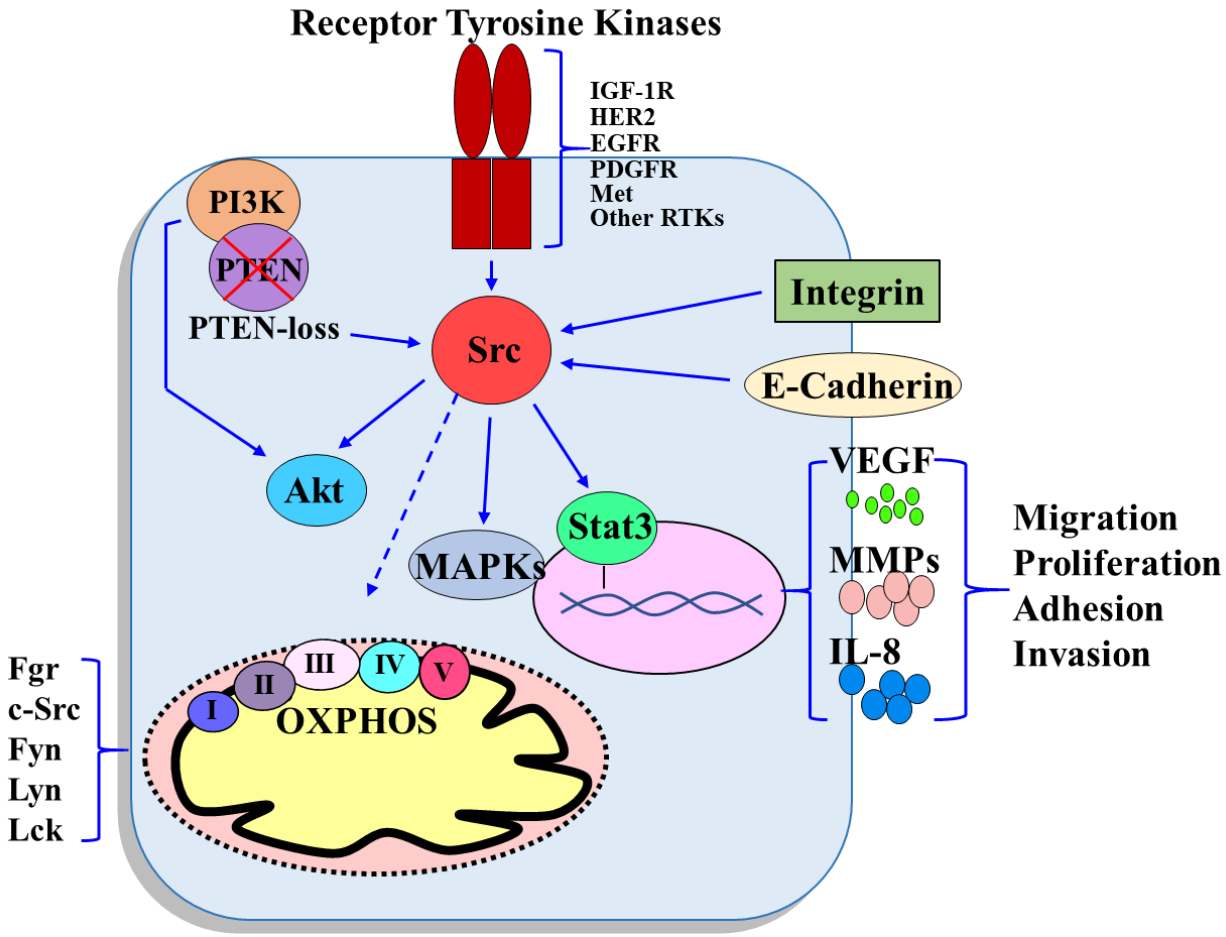


**Figure 3. Src signaling pathways.** Src interacts with multiple receptor tyrosine kinases (RTKs) including insulin-like growth factor-1 receptor (IGF-1R), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and c-Met (Met), and activated their downstream signaling to promote cell survival. Additionally, Src is also activated by these RTKs as well as integrins and the erythropoietin receptor (E-cadherin), and other downstream signaling events such as the loss of phosphatase and tensin homolog (PTEN). The activation of Src leads to other cellular signaling events including the activation of Akt for cell growth and proliferation, the activation of signal transducer and activator of transcription 3 (STAT3), and upregulation of secretary factors that are involved in metastasis, migration, proliferation, adhesion and invasion. Matrix metalloproteinases (MMPs) promote the breakdown of the extracellular matrix for the invasion of the surrounding tissue, while the production of interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF), signaling molecules that promote tumor angiogenesis, are increased by c-Src activation. Five of the Src family kinase members (Fgr, c-Src, Fyn, Lyn, and Lck) are translocated into the mitochondria where they modulate cellular energy metabolism by the phosphorylation of key enzymes involved in metabolic pathways, including oxidative phosphorylation (OXPHOS). Figure partially adapted from (Roskoski, 2015; Yeatman, 2004; S. Zhang & Yu, 2012).

## 2.3. Organization of SFKs

The human c-Src gene encodes a protein that is 536 amino acids long, roughly 52-62 kilodaltons (kDa), while the chicken c-Src gene encodes a protein that has 533 residues and exhibits 99.6% sequence identity to the human c-Src gene with the largest variation near the N-terminus (Roskoski, 2015). Interestingly, c-Src, Yes, and Fyn have a high sequence homology of roughly 77-84% identity in their conserved regions (E. C. Koc et al., 2016) with an even higher percentage of sequence identity in their kinase domains, described below. Their high sequence homology causes them to have overlapping functions that make them redundant and able to compensate for the loss of each other to maintain cellular functions (Thomas et al., 1995). Although many studies have been performed with the human c-Src kinase, the numbering system from the chicken c-Src kinase is historically used in most of the literature and will be used in the remainder of this dissertation, unless otherwise stated.

### 2.3.1. SFK Domain Organization

The SFKs display similar kinase structure and domain organization with over 70% sequence identity between SFK members (Brown & Cooper, 1996). Each kinase is composed of six distinct regions, from the N- to C- terminus: 1) the N-terminal 14-carbon myristoyl group attached to the Src homology (SH) 4 domain, 2) the unique region, 3) the SH3 domain, 4) the SH2 domain, 5) the protein kinase domain (SH1) which is attached to the SH2 domain by a linker region, and 6) the C-terminal regulatory tail (Boggon & Eck, 2004; Brown & Cooper, 1996), as shown in Fig 4A.

The SH4 domain contains a short N-terminal membrane anchor that encompasses a seven N-terminal amino acid residue chain and a myristoylation site (or palmitylation in some SFKs) (Resh, 1993; Thomas & Brugge, 1997), which is required for the attachment of the SFKs to

cellular membranes and kinase activity (Brown & Cooper, 1996; Kaplan, Mardon, Bishop, & Varmus, 1988; Roskoski, 2015; Schultz, Henderson, Oroszlan, Garber, & Hanafusa, 1985; Thomas & Brugge, 1997). Following the SH4 domain is the unique domain which is composed of 50 to 70 poorly conserved amino acids and is distinct for each family member. It has been proposed that this unique region is important for the interactions of certain family members with specific receptors or proteins.

The next three domains, the SH3, SH2, and the protein kinase domain (SH1), are important for the modular structure of the Src family kinases. The SH3 and SH2 domains are protein-binding domains that are found in lipid kinases, protein and lipid phosphatases, adaptor molecules, transcription factors, cytoskeletal proteins, and other proteins, and aid in the binding of SFKs with their adaptor proteins (B. J. Mayer & Baltimore, 1993; Roskoski, 2004; Thomas & Brugge, 1997). The SH3 domain binds to proteins containing proline rich motifs (PXXP consensus), which is important for inter- and intramolecular interactions that recruit substrates, regulate the catalytic activity, and localize Src kinases (Cohen, Ren, & Baltimore, 1995; Rickles et al., 1995; Thomas & Brugge, 1997), while the SH2 domain binds to phosphotyrosine sites of other proteins, preparing the SFKs for activity. Fyn, c-Src, Lck, and Fgr, have preferences to interact with other proteins that contain the phosphotyrosine motif (pYEEI) compared to other sequences (B. A. Liu, Engelmann, & Nash, 2012; Superti-Furga, 1995).

The SH1 or kinase domain is made of two lobes that form the catalytic cleft. This domain provides the enzymatic activity for the SFKs (Brown & Cooper, 1996; Roskoski, 2004; Superti-Furga, 1995) and contains one of the most important Tyr phosphorylation sites, Tyr416, while the second, Tyr527, resides in the short C-terminal tail that follows the kinase domain. The two

regulating Tyr residues, Tyr416 and Tyr527, are highly conserved among all SFKs, especially between c-Src, Yes, and Fyn (Fig. 4B).

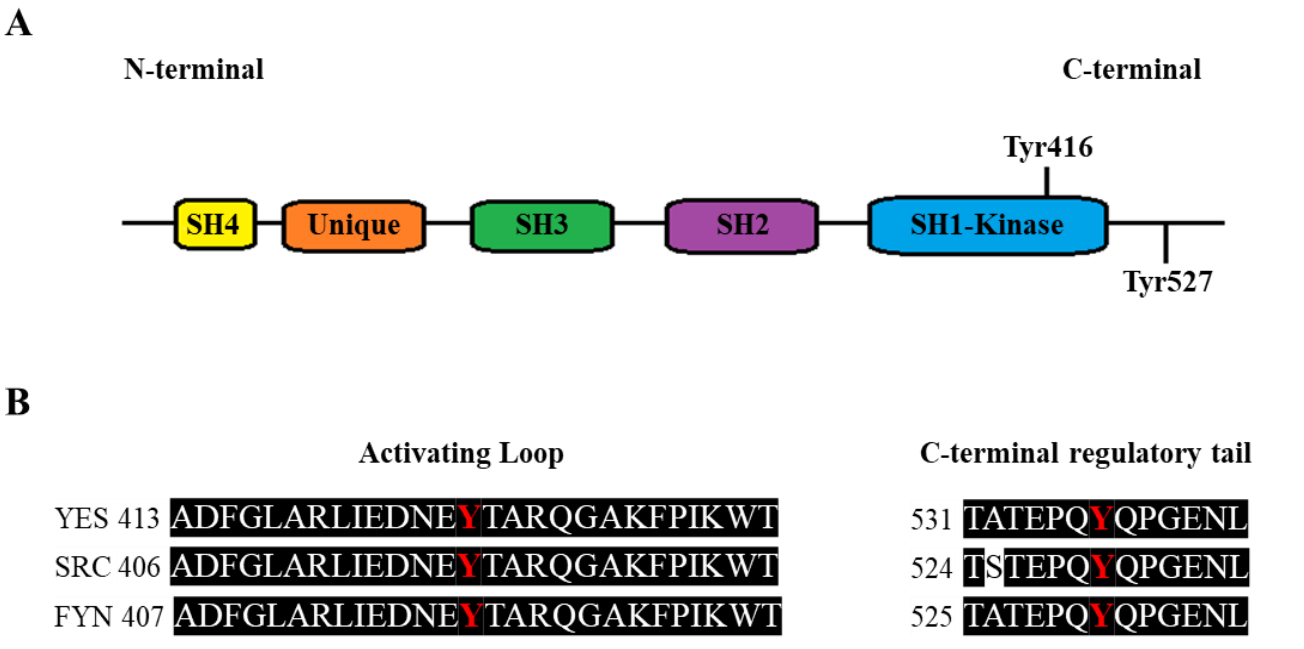


**Figure 4. Src family kinase domain structure. A)** Six domains compose the structure of the kinases and are lined from the N-terminal to the C-terminal: the Src homology 4 (SH4) domain (yellow), the unique region (orange), the SH3 domain (green), the SH2 domain (purple), the SH1 or kinase domain (blue) which contains the conserved tyrosine residue (Tyr416) involved in the activation and autophosphorylation of SFKs, and the C-terminal regulatory tail which encompasses a second conserved tyrosine residue (Tyr527) that is responsible for keeping the SKFs in the inactive state. **B)** The conservation between YES, SRC, and FYN at the activating loop and the C-terminal regulatory tail. The activating and inhibiting tyrosine residues, Tyr416 and Tyr527, respectively, are shown in red.

### 2.3.2. Activation and Inactivation of SFKs

The activity of SFKs is mainly regulated by the phosphorylation and dephosphorylation of the two major Tyr residues, Tyr416 and Tyr527. Similar to many Tyr kinases, SFKs are kept in the inactive state 90-95% of the time (Roskoski, 2015; Salvi et al., 2005; Zheng, Resnick, & Shalloway, 2000). Under basal conditions, Tyr527 is phosphorylated (pTyr527), binds to the SH2 domain by intramolecular interactions, and forms a salt bridge. This stabilizes the enzyme in a dormant or "closed" conformation and inhibits the ability for external ligands to interact with the SH3 or SH2 domains (Brown & Cooper, 1996; Roskoski, 2004; Thomas & Brugge, 1997). In the closed conformation, Tyr416 is sequestered so it is inaccessible to other kinases and unable to be phosphorylated, preventing kinase activation (Fig 5A). Both Csk (C-terminal Src kinase) or Chk kinases are responsible for the phosphorylation of Tyr527 and inactivation of SFKs (Okada, 2012; Okada & Nakagawa, 1989; Zrihan-Licht et al., 1997).

The activation of SKFs occurs in two steps. In the first step, protein tyrosine phosphatases, such as the protein tyrosine phosphatase alpha (PTPα), phosphoenolpyruvate phosphatase (PEP), and Src homology 2 domain-containing protein tyrosine phosphatase 1 and 2 (SHP-1 and SHP-2), displace pTyr527 from the SH2 domain pocket and mediate the dephosphorylation of Tyr527 (Zheng et al., 2000; Zheng, Wang, & Pallen, 1992). During the second step, the enzymatic catalytic cleft opens and exposes Tyr416 to autophosphorylation (Brown & Cooper, 1996; Huse & Kuriyan, 2002; Xu, Doshi, Lei, Eck, & Harrison, 1999). Tyr416 phosphorylation stabilizes the enzyme in the active conformation and allows for continued phosphorylation and activation of SFKs by other Tyr kinases (Fig 5B).

SFKs are also redox-sensitive and can be activated by ROS, including hydrogen peroxide ($H_2O_2$) and peroxynitrite (Akhand et al., 1999). In fact, ROS activation of SFKs leads to a third,

highly active conformation (Giannoni, Buricchi, Raugei, Ramponi, & Chiarugi, 2005; Giannoni, Taddei, & Chiarugi, 2010; Krasnowska et al., 2008). SFKs contain two conserved, redox-regulated cysteine (Cys) residues at 245 and 487 (Cys245 and Cys487) which are located in the SH2 and kinase domains, respectively (Giannoni & Chiarugi, 2014; Giannoni et al., 2010). When the SFK is activated by ROS, these two Cys residues become oxidized, resulting in the formation of an intramolecular disulfide bond between the SH2 and kinase domain, to protect the dephosphorylation of Tyr416 and keep the kinase in a highly active conformation (Fig 5C). The constitutively active kinases can cause significant defects in downstream signaling and can result in cytoskeletal rearrangement and cell spreading, leading to physiological disturbances and the development of various diseases, such as cancer (Giannoni et al., 2005).

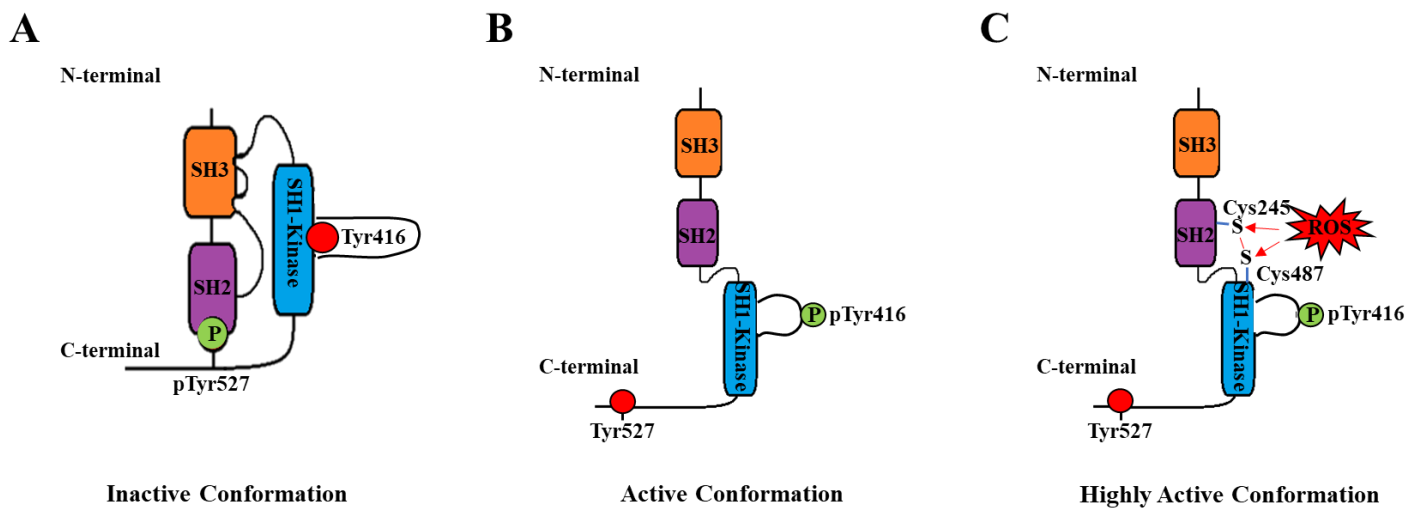


**Figure 5. Activation of Src family kinases. A)** Resting cells exposed to normal environmental conditions exhibit an inactive (closed) conformation, characterized by intramolecular interactions between the SH2 domain and the phosphorylated Tyr527 and associations between the SH3 and linker domain, sequestering Tyr416 in its dephosphorylated state. **B)** Once Src is activated in the early stage of cell adhesion or by growth factor stimulations, phosphorylated Tyr527 (pTyr527) is dissociated from the SH2 domain and dephosphorylated by several protein tyrosine phosphatases. The catalytic cleft opens and Tyr416 is autophosphorylated by another Src kinase molecule, stabilizing the enzyme in its active conformation and allowing for continued phosphorylation and activation. **C)** During high ROS generation, Src is oxidized in one or both of the redox-regulated cysteine residues, Cys245 and Cys487, located in the SH2 domain and kinase domain, respectively, resulting in the formation of an intramolecular disulfide bond (S-S) between the SH2 and kinase domain, keeping the Src kinase in a highly active conformation. Activating Tyr residue (green); inactivated Tyr residue (red) (see text for details). Figure partially adapted from (Giannoni & Chiarugi, 2014).

## 2.4. Src Family Kinases and the Mitochondria

### 2.4.1. Src Kinases are Translocated into the Mitochondria

Recently, five of the SFK members, c-Src, Fyn, Lyn, Fgr, and Lck, along with their regulator kinase Csk, have been found in the intermembrane space and inner membrane of the mitochondria in rat brain tissues and cell lines (Miyazaki et al., 2003; Salvi et al., 2002; Salvi et al., 2005; Vahedi et al., 2015). Additionally, they are activated via phosphorylation by a variety of mitochondrial signals, such as redox state, ATP level, hypoxia, and calcium concentration (Hebert-Chatelain, 2013; Hofer & Wenz, 2014; Sato et al., 2005), demonstrating their significance in the regulation of mitochondrial functions. Although some studies suggested that these SFKs permanently reside in the mitochondria (Salvi et al., 2005), other studies demonstrated continuous import and export of SFKs into and out of the mitochondria (Boerner, Demory, Silva, & Parsons, 2004; Demory et al., 2009).

Since SFKs lack a mitochondrial localization sequence, they rely on interactions with adaptor proteins to be translocated into the mitochondria. The C-terminal proline-rich motif in the SH3 domain of SFKs was established as a key player in directing the SFKs to their final destination, which is carried out by the presence of a localization signal on the SFK interacting partners. Several proteins including the protein kinase A (PKA) anchoring protein 121/84 (AKAP121/84) and the protein tyrosine phosphatase D1 (PTPD1) (Cardone et al., 2004; Livigni et al., 2006), the human T-cell leukemia virus type 1 accessory protein p13 (Tibaldi et al., 2011), and the downstream of Tyr kinase docking protein 4 (Dok-4) (Itoh et al., 2005), are involved in the translocation of Src kinases into the mitochondria, further highlighting the importance of these SFKs within the mitochondria.

### 2.4.2. Mitochondrial Energy Metabolism is Regulated by SFKs

SFK phosphorylation impacts the function of many mitochondrial proteins, including those involved in metabolic pathways that feed into and regulate mitochondrial OXPHOS and energy metabolism (Fig. 6). In fact, several enzymes involved in carbohydrate metabolism, pyruvate decarboxylation, and the TCA cycle, have been identified as SFK targets. Mitochondrial translation components, responsible for the synthesis of the 13 core OXPHOS proteins, as well as the subunits of OXPHOS complexes I-V were also identified as targets for SFK phosphorylation, summarized in Table 2.

Src family kinases are implicated in the regulation of carbohydrate metabolism (glycolysis) through the phosphorylation of key enzymes such as glycerol-3-phosphate dehydrogenase, which links carbohydrate and lipid metabolism, and hexokinases 1 and 2 (HK1 and HK2), which catalyzes the phosphorylation of glucose to glucose-6-phosphate (Lewandrowski et al., 2008). Although the physiological role of the phosphorylation of glycerol-3-phosphate dehydrogenase is unknown, the phosphorylation of HK1 and HK2 by c-Src kinase was shown to decrease their activities and significantly impair mitochondrial OXPHOS (J. Zhang et al., 2017). c-Src has also been shown to regulate pyruvate decarboxylation by phosphorylating PDH and inhibiting its activity (Y. Jin et al., 2016; Lewandrowski et al., 2008; J. Zhang et al., 2017). Furthermore, the first two steps of the TCA cycle were found to be phosphorylated by Src kinases. Citrate synthase (CS) and aconitase are phosphorylated by SFKs (Lewandrowski et al., 2008; Salvi et al., 2007); however, the physiological relevance of these modifications and their effects on energy metabolism is not currently known. Interestingly, aconitase is also implicated in the stabilization of mtDNA and iron metabolism; therefore, the

phosphorylation of aconitase by Fgr at Tyr71, Tyr544, and Tyr665 (Salvi et al., 2007), could have multiple effects on metabolism, yet these effects remain to be elucidated.

Mitochondrial translation components and, therefore, the expression of OXPHOS complexes, are also regulated by SFKs. Recently, our group has demonstrated the association of Fyn kinase with mitochondrial translation machinery, MRPs, and showed an induction of mitochondrial protein synthesis and OXPHOS expression in liver cancer cell lines (E. C. Koc et al., 2016). On the contrary, Lck kinase was also reported to interact with mitochondrial translation machinery by competitively binding to CR6-interacting factor (CRIF1), a mitochondrial ribosomal protein (E. C. Koc et al., 2013), and inhibiting OXPHOS expression and activity (Vahedi et al., 2015). Since mitochondrial translation is responsible for the synthesis of the 13 essential OXPHOS subunits, SFK phosphorylation plays a clear role in regulating energy metabolism.

In fact, SFKs have also modulated the activity of OXPHOS complexes by directly phosphorylating their subunits (described in Table 2). Interestingly, the first identified mitochondrial protein phosphorylated by c-Src kinase was COII, a mitochondrial-encoded subunit of OXPHOS complex IV (Miyazaki et al., 2003). This phosphorylation event was the first to demonstrate the regulation of OXPHOS activity by Src-dependent phosphorylation of OXPHOS complexes. Since then, SFKs have been shown to phosphorylate several subunits from OXPHOS complexes I-V (Augereau et al., 2005; Hebert-Chatelain et al., 2012; Lewandrowski et al., 2008; Ogura, Yamaki, Homma, & Homma, 2012; Salvi et al., 2007), summarized in Fig. 6. The 39 kDa and 75 kDa subunits, NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2), and NADH:ubiquinone oxidoreductase subunit B10 (NDUFB10) of complex I were identified as SFK targets (Augereau et al., 2005; Hebert-Chatelain et al., 2012; Ogura et al.,

2012). Although the physiological relevance of the phosphorylation of the 39 kDa and 75 kDa subunits remains to be identified, the phosphorylation of the core subunit, NDUFV2, and NDUFB10 by c-Src is required for the NADH activity and, therefore, increases complex I activity resulting in stimulated OXPHOS function. Additionally, succinate dehydrogenase subunit A (SDHA) of complex II, subunits I and II of complex III, and subunits α, β, γ, and ε of complex V were also phosphorylated by SFKs, with the phosphorylation of complex III and V leading to a decrease in their complex activities (Augereau et al., 2005; Hebert Chatelain, Dupuy, Letellier, & Dachary-Prigent, 2011; Lewandrowski et al., 2008).

The phosphorylation of COII by c-Src induced the enzymatic activity of complex IV in osteoclasts (Miyazaki et al., 2003; Miyazaki, Tanaka, Sanjay, & Baron, 2006). However, when COII was phosphorylated in combination with the epidermal growth factor receptor (EGFR), the activity of OXPHOS complex IV was significantly reduced (Boerner et al., 2004; Demory et al., 2009) implying further studies should be performed to identify the effects of c-Src on COII in health and disease. In addition to regulating OXPHOS complexes, SFKs also regulate energy metabolism by targeting the ATP/ADP antiporters, adenine nucleotide translocase 1 and 2 (ANT1 and ANT2), which are involved in transporting ADP to the ATPase (complex V) and providing ATP to the cell by exporting it from the mitochondria (Lewandrowski et al., 2008), although the effect of SFK phosphorylation on ANT1 and ANT2 are not yet known. Together, these studies clearly demonstrate the regulation of mitochondrial energy metabolism through the phosphorylation of mitochondrial proteins by SFKs in health and disease.

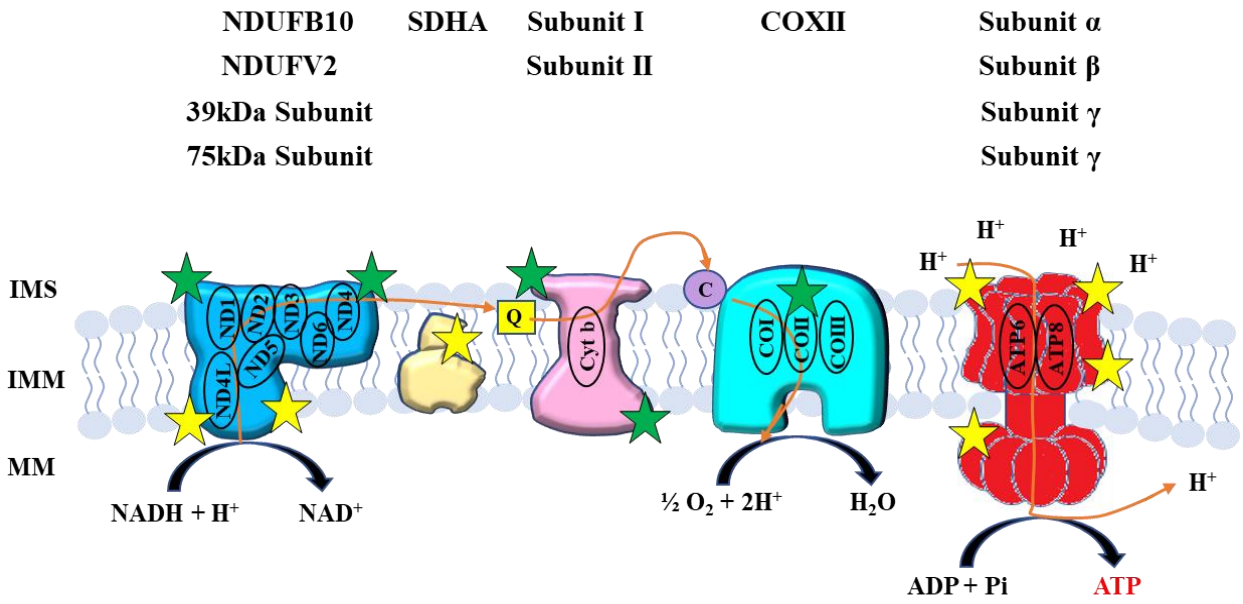


**Figure 6. OXPHOS complex subunits phosphorylated by Src family kinases.** Subunits of oxidative phosphorylation complexes are phosphorylated by Src family tyrosine kinases to alter ATP production. The yellow stars represent phosphorylation sites of SFKs that have not been identified, and the green stars represent Tyr phosphorylation sites of c-Src kinase.

In addition to energy metabolism, apoptosis is also established as a major factor in mitochondrial function. Interestingly, SFKs also regulate different mitochondrial-dependent apoptotic pathways. Lck is involved in the activity of the apoptotic signal transduction pathways after irradiation and ceramide exposure (Belka et al., 1999; Hur, Yun, & Won, 2004; Manna & Aggarwal, 2000), and has, therefore, been identified as a positive regulator of apoptosis (Samraj, Stroh, Fischer, & Schulze-Osthoff, 2006). Lyn was suggested to contribute to apoptosis after exposure to radiation and the deprivation of growth factors (Qin, Minami, Kurosaki, & Yamamura, 1997; Uckun et al., 1996), implicating its role in pro-apoptotic events; however, Lyn was also suggested to preserve mitochondrial integrity and protect against apoptosis during liver regeneration (Ferri et al., 2005; F. Y. Lee et al., 1999). Other SFKs, such as c-Src, also possess anti-apoptotic properties (Lluis, Buricchi, Chiarugi, Morales, & Fernandez-Checa, 2007). Therefore, a better understanding of how these SFKs regulate mitochondrial functions including OXPHOS and apoptosis is essential for disease prevention.

| Pathway | Target | Residue | Kinase | Alteration | References |
|---|---|---|---|---|---|
| **Carbohydrate metabolism (glycolysis)** | Glycerol-3-phosphate dehydrogenase | Unidentified | Src kinases | Unknown | (Lewandrowski, Sickmann et al. 2008) |
| | Hexokinases 1 and 2 | Tyr732 and Tyr686 | c-Src kinase | Decreases activity of HK1 and HK2 | (Lewandrowski, Sickmann et al. 2008, Zhang, Wang et al. 2017) |
| | Pyruvate dehydrogenase | Tyr289 | c-Src kinase | Decreases activity of PDH | (Jin, Cai et al. 2016) |
| **TCA cycle/CAC** | Aconitase Hydratase | Tyr71, Tyr544, Tyr665 | Fgr kinase | Unknown | (Salvi, Morrice et al. 2007) |
| | Citrate Synthase | Unidentified | Src kinases | Unknown | (Lewandrowski, Sickmann et al. 2008) |
| **Protein Synthesis** | Ribosomal Proteins | Unidentified | Fyn kinase | Increases protein synthesis | (Koc, Miller-Lee et al. 2016) |
| | CR6-interacting factor 1 | Unidentified | Lck kinase | Decreases protein synthesis | (Vahedi, Chueh et al. 2015) |
| **OXPHOS complex I** | 39kDa and 72kDa | Unidentified | Src kinases | Unknown | (Augereau, Claverol et al. 2005) |
| | NDUFV2 | Tyr193 | c-Src kinase | Increases activity of CI | (Hebert-Chatelain, Jose et al. 2012) |
| | NDUFB10 | Unidentified | c-Src kinase | Increases activity of CI | (Hebert-Chatelain et al., 2012; Ogura et al., 2012) |
| **OXPHOS complex II** | SDHA | Unidentified | Src kinases | Unknown | (Augereau et al., 2005; Ogura et al., 2012; Salvi et al., 2007) |
| **OXPHOS complex III** | Subunits I and II | Unidentified | Src kinases | Decreases activity of CIII | (Augereau et al., 2005; Hebert Chatelain et al., 2011) |
| **OXPHOS complex IV** | COII | Unidentified | c-Src kinase | Increases activity of CV | (Augereau et al., 2005; Miyazaki et al., 2003; Miyazaki et al., 2006) |
| | COII | Unidentified | c-Src kinase and EGFR | Decreases activity of CIV | (Demory, Boerner et al. 2009, Boerner, Demory et al. 2004) |
| **OXPHOS complex V** | α-, β-, γ-, and ε- | Unidentified | Src kinases | Decreases activity of CV | (Augereau et al., 2005; Hebert-Chatelain et al., 2012; Hebert Chatelain et al., 2011; Lewandrowski et al., 2008) |
| **Apoptosis (MPTP)** | ATP/ADP translocase 1 and 2 | Unidentified | Src kinases | Unknown | (Lewandrowski, Sickmann et al. 2008) |
| | Creatine kinase | Unidentified | Src kinases | Unknown | (Lewandrowski, Sickmann et al. 2008) |

**Table 2. Mitochondrial pathways that are regulated by Src family kinases.** Individual targets, modified residues, the respective modifying Src family kinase, and the physiological role from each modification is listed.

## 2.5. SFK Signaling and Cancer

Alterations in the expression of SFKs have been shown to contribute to the initiation and progression of multiple diseases including neurological disorders (Alzheimer's and Parkinson's disease), metabolic syndrome, diabetes, and various types of cancer (Elias & Ditzel, 2015; Irby & Yeatman, 2000; Lu et al., 2009; K. Yang et al., 2011; Yeatman, 2004). Many SFK members have been established and characterized as proto-oncogenes (Brown & Cooper, 1996; Czernilofsky et al., 1980; Irby & Yeatman, 2002; L. C. Kim, Song, & Haura, 2009; M. P. Kim, Park, Kopetz, & Gallick, 2009; Stehelin et al., 1976). Surprisingly, to date, no somatic mutations have been identified in SFK genes (Frame, 2004; Yeatman, 2004); therefore, dysregulation of their activities play critical roles in the etiology of cancer. In fact, aberrant activation is implicated in disease development, growth, and progression due to the induction of multiple malignant properties of SFKs including proliferation, invasion, angiogenesis, and metastasis (Elsberger, 2014; M. P. Kim et al., 2009; Yeatman, 2004), linking SFKs to both tumorigenicity and metastasis (Brown & Cooper, 1996; Irby & Yeatman, 2000). c-Src kinase is the SFK member most often implicated in cancer and increased expression and/or activity has been reported in a variety of human cancers including ovarian, lung, breast, colorectal, pancreatic, brain, gastric, liver, melanoma, and leukemia (Dai et al., 1998; Irby & Yeatman, 2002; Rosen et al., 1986; Yeatman, 2004). Aberrant expression and activity of c-Src has been positively correlated with cancer stage, disease recurrence, and adverse prognosis, and is frequently associated with increased cancer metastasis and resistance to treatments (Elsberger, 2014; Irby & Yeatman, 2000; E. L. Mayer & Krop, 2010; Yeatman, 2004; S. Zhang et al., 2011). These findings demonstrate the need for a better understanding of the involvement of c-Src in cancer types, which can be used for the development of novel treatments.

Liver cancer is one of the most common cancer types that is increasing due to the rising rates of obesity and metabolic syndrome and is the sixth major cause of cancer mortality in men (Walker et al., 2019). Approximately 85-90% of primary liver cancers occurring are hepatocellular carcinoma (HCC) (El-Serag & Rudolph, 2007; Ren, Fang, Ding, & Chen, 2016; Torre et al., 2015). HCC is one of the most metastatic and malignant cancers and is strongly associated with poor prognosis, recurrence, and resistance to treatments (Daher, Massarwa, Benson, & Khoury, 2018; Pang et al., 2008; Ren et al., 2016). Due to the high recurrent and metastatic nature of HCC, the five-year survival rate is low (R. Zhao et al., 2015); therefore, the identification of oncogenes involved in HCC progression are essential to the prevention and treatment.

The overexpression and/or increased activity of c-Src has been found in HCC (Masaki et al., 1998; Sun et al., 2008; R. Zhao et al., 2015). Aberrant c-Src activity was suggested to contribute to the malignant transformation of hepatocytes and the poor prognosis of patients (Masaki et al., 1998). Interestingly, oxidative stress, which is strongly associated with mitochondrial dysfunction, was indicated as a contributor to HCC carcinogenesis (Hsu, Lee, & Wei, 2013; Klaunig & Kamendulis, 2004; Kumar, Zhao, & Wang, 2011), and is an activator of c-Src kinase. In fact, c-Src has been shown to alter mitochondrial function and energy metabolism by phosphorylating mitochondrial proteins and is implicated in promoting the Warburg effect and malignant properties in various cancer types (Y. Jin et al., 2016; Wallace, 2012; J. Zhang et al., 2017). Previous studies have shown c-Src phosphorylation of HK1 and HK2 at Tyr732 and Tyr686, respectively, in addition to PDH at Tyr289 in cancer cells which inhibited their activities, promoted the glycolytic flux and increased glycolysis, impaired OXPHOS function and ATP synthesis, and contributed to the Warburg effect and the

development of cancer (Y. Jin et al., 2016; J. Zhang et al., 2017). Additionally, c-Src promoted the metastatic phenotype by increasing cell proliferation and enhancing angiogenesis (Fleming et al., 1997; W. Mao et al., 1997; Munshi et al., 2000) as well as stimulating the cell cycle progression to prevent apoptosis by increasing the expression of B-cell lymphoma-extra-large (Bcl-XL) (Karni, Jove, & Levitzki, 1999) and activating Stat3 (Bromberg et al., 1999; Catlett-Falcone et al., 1999).

c-Src plays a critical role in cell growth and maintenance; however, the overactivation of c-Src leads to malignant phenotypes found in cancer. The presence of c-Src and other SFKs within the mitochondria in cancer cells not only implies their role in mitochondrial dysfunction and cancer development (Demory et al., 2009; Hebert-Chatelain et al., 2012; E. C. Koc et al., 2016), but it also demonstrates that mitochondrial metabolism is essential to cancer cell growth, especially since mitochondria are also essential for the biosynthetic machineries of lipids, proteins, and nucleic acids which are largely derived from the TCA cycle. Therefore, a better understanding of the role of c-Src, and other SFKs, in mitochondrial energy metabolism in different cancer cell types needs to be established. Furthermore, identifying direct pathways and substrates regulated by SFKs can make them pharmacological tools for drug development to allow for improved mitochondrial function. Aberrant expression of c-Src was clearly implicated in the progression of HCC, which is also associated with high oxidative stress. Yet, the role of c-Src on mitochondrial dysfunction in liver cancer remains to be elucidated.

### 2.6. SFKs as Drug Targets

Due to the increased expression of SFKs in a large number of human cancers, they have become promising targets for cancer therapies (Blake et al., 2000; Hanke et al., 1996; Roskoski, 2015; S. Zhang & Yu, 2012). To date, only five orally effective multi-kinase inhibitors have

been approved by the Food and Drug Administration (FDA). In fact, Vandetanib and Sarcatinib were initially developed as SFK inhibitors, while other inhibitors such as Bosutinib, Dasatinib, and Ponatinib were developed as multi-kinase inhibitors and demonstrated to target SFKs (Boschelli et al., 2004; Hennequin et al., 2006; W. S. Huang et al., 2010; Lombardo et al., 2004; Nowak et al., 2007). Although Vandetanib, Ponatinib, and Sarcatinib were shown to target all SFKs, Bosutinib and Dasatinib were shown to target specific SFKs c-Src, Lyn, and Hck and c-Src, Lck, Fyn, and Yes, respectively. These inhibitors have been used to target specific cancer types, such as small cell lung, colorectal, and ovarian cancers; however, they are currently undergoing additional clinical trials to be used for the treatments of other cancers.

In addition to these FDA-approved drugs for cancer treatments, other SFK inhibitors such as 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2) and 2,3-Dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide (SU6656) have been evaluated for their potential use in cancer treatments (Blake et al., 2000; Finn et al., 2013; Hanke et al., 1996; Lau et al., 2009; E. L. Mayer & Krop, 2010). PP2 and SU6656 are small molecule inhibitors that block the ATP binding site of the ubiquitously expressed SFK members c-Src, Yes, Fyn, and Lyn (Blake et al., 2000; Hanke et al., 1996). While these inhibitors have been proven effective in reducing cell proliferation, and decreased cancer progression by suppressing SFK activity (Finn et al., 2013; H. Guo et al., 2016; Zhu et al., 2018), clinically significant data is limited. Unfortunately, breast cancer, HCC, and others are highly metastatic and resistant to conventional cancer treatments, only providing the ability to minimally extend the life expectancy of patients (E. L. Mayer & Krop, 2010; Salem, Whitaker-Menezes, Howell, Sotgia, & Lisanti, 2012; Wu et al., 2018; S. Zhang et al., 2011; R. Zhao et al., 2015). Currently, the multi-kinase inhibitor Sorafenib is the only approved drug for the systemic

treatment of HCC; however, it only extends the life of patients by 3-4 months (Daher et al., 2018; Llovet et al., 2008; Wilhelm et al., 2008), demonstrating a need for alternative treatment options.

### 2.6.1. Natural Antioxidants as SFK Inhibitors

Increased levels of ROS have been found in various cancer types and are often associated with mitochondrial OXPHOS dysfunction (Delmas, Jannin, Cherkaoui Malki, & Latruffe, 2000; H. Guo et al., 2016; Liao et al., 2010; Zhu et al., 2018). Due to high levels of ROS in cancer and the ability of ROS to activate SFKs, natural antioxidants such as kaempferol and resveratrol have been evaluated as chemotherapeutic agents and Src kinase inhibitors. Kaempferol and resveratrol are both naturally occurring antioxidants that have anti-tumor, anti-inflammatory, and chemo-preventative pharmacological activities (H. Guo et al., 2016; Y. B. Huang et al., 2014; Liao et al., 2010; M. Singh, Kaur, & Silakari, 2014; Udenigwe, Ramprasath, Aluko, & Jones, 2008; Zhu et al., 2018). Kaempferol belongs to the flavonoid family that also has anti-diabetic effects (A. Y. Chen & Chen, 2013). Our laboratory has previously demonstrated increased mitochondrial complex II activity with kaempferol treatment, demonstrating improved mitochondrial function (Cimen et al., 2010). Additionally, previous studies demonstrated reduced cell proliferation, migration, invasion, and apoptosis in the liver cancer cell line, HepG2, when cells were treated with kaempferol (H. Guo et al., 2016; Zhu et al., 2018). Resveratrol is a polyphenol that has many protective effects against cardiovascular disease, age-related deterioration, and cancer (Baur, 2010; Jang et al., 1997). Resveratrol has been shown to reduce cell growth and proliferation by inhibiting the cell cycle and inducing apoptosis in various cancer types including liver and breast cancer (Delmas et al., 2000; Kocsis, Marcsek, Jakab, Szende, & Tompa, 2005; Liao et al., 2010; Nakagawa et al., 2001). Resveratrol induces apoptosis by stimulating the

production of intracellular ROS to activate the ROS-dependent apoptotic pathways (Miki et al., 2012). Similarly, resveratrol treatments have also been reported to increase mitochondrial function in cancer cells (Haohao, Guijun, Juan, Wen, & Lulu, 2015; Lagouge et al., 2006). Interestingly, both kaempferol and resveratrol have been shown to have inhibitory effects on Src pathways (K. M. Lee et al., 2010; Oz et al., 2019). Kaempferol acts similarly to PP2 and SU6656 by blocking the ATP binding site of Src kinases, inhibiting the activity, and preventing downstream signaling (K. M. Lee et al., 2010). Although these two antioxidants show promise as SFK inhibitors, their effects on mitochondrial OXPHOS and energy metabolism remains to be elucidated. Due to the aberrant expression of c-Src and its regulatory role in the metabolic reprogramming of cancer cells by altering mitochondrial function, understanding the impact of these inhibitors on mitochondrial energy metabolism will prove to be therapeutically beneficial.

## 2.7. Research Specific Aims and Hypotheses

### Specific Aims

Mitochondrial translation is responsible for producing 13 essential subunits of oxidative phosphorylation (OXPHOS) complexes in mammals. The mitochondria produce the majority of the cell's energy by OXPHOS; therefore, protein synthesis is critical for mitochondrial energy production. Mitochondrial dysfunction is often found to be associated with obesity, type 2 diabetes (T2D), and cancer (Kelley et al., 2002; J. A. Kim et al., 2008; Wallace, 1999, 2012). Although several studies have demonstrated diminished mitochondrial energy metabolism in obesity and T2D subjects, the mitochondrial molecular pathogenesis of the disease remains to be discovered. Sequence variants in mitochondrial genes involved in mitochondrial protein synthesis, such as *Dars2* and several mitochondrial proteins, along with variants in OXPHOS complex subunits, *Sdhc* and *Ndufs2*, have been identified in the human obesity and type 2

diabetes model, TALLYHO/Jng (TH) mice. For this reason, **impaired mitochondrial protein synthesis and oxidative energy metabolism results in mitochondrial dysfunction which can contribute to the development of obesity and T2D in TH mice.**

Post-translational modifications (PTMs) have been shown to be a regulatory mechanism for many mitochondrial processes; however, the regulation of mitochondrial OXPHOS complex expression and energy metabolism remains to be elucidated. The Src family kinases (SFKs) c-Src and Fyn are expressed in all cell types and have been located within the mitochondria where they are involved in the phosphorylation and regulation of mitochondrial proteins (Hebert-Chatelain, 2013; Miyazaki et al., 2006; Salvi et al., 2005). These kinases are activated by cellular reactive oxygen species (ROS), a byproduct of OXPHOS (Giannoni et al., 2005). In fact, in one of the most common and malignant cancers, human hepatocellular carcinoma (HCC), increased c-Src expression and activity was reported and correlated with disease progression and cancer metastasis (Masaki et al., 1998; Zhao et al., 2015). Although c-Src phosphorylates many mitochondrial proteins, its role in the regulation of mitochondrial energy metabolism in health and disease, such as liver cancer, has yet to be established. **Therefore, due to the essential role of mitochondria in energy metabolism, deregulation of mitochondrial OXPHOS complex expression by increased c-Src expression and activation may result in dysfunctional energy metabolism.**

Additionally, elevated Fyn kinase expression has been reported in liver cancer and shown to alter mitochondrial energy metabolism by stimulating mitochondrial protein synthesis (E. C. Koc et al., 2016). Hyperactivation of Fyn, along with other SFKs, has contributed to the development and progression of metabolic diseases such as neurodegeneration and cancer. However, many patients are becoming resistant to available treatment options due to the

increased activity of SFKs, leading to investigations in alternative treatments such as natural antioxidants. Therefore, a better understanding of Fyn kinase in cellular signaling and the regulation of mitochondrial energy metabolism is necessary for the development of novel therapeutic strategies to improve disease prognosis. For this reason, **the inhibition of Fyn kinase activity may improve mitochondrial function and energy metabolism and slow metabolic disease progression.** Our studies will help devise mechanisms for how c-Src and Fyn regulate mitochondrial energy metabolism and oxidative phosphorylation as well as the development of therapeutic tools for combating mitochondrial dysfunction in metabolic diseases.

**Aim 1: Determine the changes in oxidative phosphorylation and mitochondrial biogenesis in human obesity and type 2 diabetes model, TALLYHO/Jng mice.**

Previous studies have implicated mitochondrial dysfunction as an underlying factor in the development of obesity, insulin resistance, and type 2 diabetes (T2D) (Kelley et al., 2002; J. A. Kim et al., 2008; Petersen et al., 2003). Reduced energy expenditure and sequence variants in mitochondrial proteins have been reported in the human obesity and T2D model TALLYHO/Jng (TH) mice (Denvir et al., 2016; X. Mao, Dillon, McEntee, Saxton, & Kim, 2014), implying that TH mice may have impaired mitochondrial function. **We hypothesize** that the mitochondrial sequence variants identified in the whole genome analysis of TH mice would affect oxidative phosphorylation and contribute to the obesity and insulin resistant phenotypes. In an effort to uncover the role of mitochondrial function in TH mice, we will investigate differences in the expression and activity of OXPHOS complexes. Additionally, the transcription and translation of nuclear- and mitochondrial-encoded OXPHOS subunits will be evaluated in mitochondrial-rich tissues (i.e. the liver and kidney) of TH and C57BL/6J (B6) mice. The role of the sequence

variants identified in TH mice will be observed and discussed. The results from this aim will identify factors that contribute to mitochondrial dysfunction in obesity and T2D and allow us to improve disease prognosis and treatment.

**Aim 2: Investigate the role of c-Src on mitochondrial OXPHOS complex expression and activity in liver cancer.**

Src family kinases (SFKs) have been shown to regulate mitochondrial energy metabolism in normal and cancer cells. In fact, dysregulation of SFK activities have been shown to contribute to the development and progression of cancer. Several studies have reported high c-Src expression and activity in human hepatocellular carcinoma (HCC) which is correlated to disease stage (Masaki et al., 1998; R. Zhao et al., 2015). c-Src kinase has also been shown to contribute to mitochondrial dysfunction and the Warburg effect (Y. Jin et al., 2016; Wallace, 2005; J. Zhang et al., 2017). **We hypothesize** that c-Src kinase expression and activity is increased in metastatic liver cancer tissues and cell lines and is associated with decreased expression of OXPHOS complex subunits, which can be reversed when treated with SFK inhibitors and c-Src siRNA. To determine the role of c-Src on mitochondrial energy metabolism in liver cancer, non-cancerous and cancerous liver biopsies from patients and common liver cancer cell lines, Hep3B and HepG2, will be evaluated for the expression of c-Src and the phosphorylation and activation of c-Src at Tyr416 (pSFK), along with changes in the expression and activity of OXPHOS complexes. Once the association between c-Src and OXPHOS complex expression is determined in the liver cancer tissues and cell lines, the cells will be treated with the SFK inhibitor PP2 and transfected with c-Src specific siRNA to observe changes in c-Src phosphorylation, OXPHOS complex expression and activity, and cell proliferation. Additionally, we will compare the results

from the liver cancer studies to studies using multiple mouse embryonic fibroblast cell lines with varying expressions of c-Src to observe if similar effects are found in normal cells. The results from this aim will reveal the correlation between c-Src and mitochondrial OXPHOS complex expression in both health and disease. The findings will also allow us to have a better understanding of how c-Src kinase alters energy metabolism and responds to SFK inhibitors to develop better treatment options for cancers with high c-Src expression and/or activity.

**Aim 3: Identify the effects of Fyn kinase inhibition with natural antioxidants on mitochondrial energy metabolism and oxidative phosphorylation in metabolic diseases.** SFK inhibitors, as well as natural antioxidants kaempferol and resveratrol which have also been reported to have inhibitory effects on Src kinase pathways (Finn et al., 2013; Lau et al., 2009; K. M. Lee et al., 2010; E. L. Mayer & Krop, 2010; Oz et al., 2019), have been investigated as potential therapies. Interestingly, Fyn kinase was shown to be a contributing factor to several metabolic disorders; however, the effects of Fyn kinase and mitochondrial energy metabolism are largely unknown. Recently, our laboratory has shown Fyn kinase is associated with and phosphorylates mitochondrial translation components and increased OXPHOS complex expression (E. C. Koc et al., 2016). **We hypothesize** that treatment of liver cancer cell lines with the SFK inhibitor, SU6656, and natural antioxidants, kaempferol and resveratrol, will impair Fyn activity and OXPHOS expression while reducing oxidative stress and decrease cell proliferation. To identify the effects of Fyn inhibition on OXPHOS complex expression, we will examine the changes in the expression of OXPHOS complexes and cell proliferation when Hep3B and HepG2 liver cancer cell lines are treated with SU6656, kaempferol, and resveratrol. The results from this aim will identify the effects of Fyn inhibition on mitochondrial oxidative

phosphorylation. These findings will also provide us with a better understanding of the effects of Fyn inhibition on mitochondrial function and cell proliferation. In addition, the data will allow us to better evaluate SU6656, kaempferol, and resveratrol as SFK inhibitors to help develop better treatment options to improve mitochondrial function and prevent the progression of metabolic disorders.

# CHAPTER 3

## MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION IS IMPAIRED IN TALLYHO MICE, A NEW OBESITY AND TYPE 2 DIABETES ANIMAL MODEL

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**Abstract**

Type 2 diabetes has become an epidemic disease largely explained by the dramatic increase in obesity in recent years. Mitochondrial dysfunction is suggested as an underlying factor in obesity and type 2 diabetes. In this study, we evaluated changes in oxidative phosphorylation and mitochondrial biogenesis in a new human obesity and type 2 diabetes model, TALLYHO/Jng mice. We hypothesized that the sequence variants identified in the whole genome analysis of TALLYHO/Jng mice would affect oxidative phosphorylation and contribute to obesity and insulin resistant phenotypes. To test this hypothesis, we investigated differences in the expression and activity of oxidative phosphorylation complexes, including the transcription and translation of nuclear- and mitochondrial-encoded subunits and enzymatic activities, in the liver and kidney of TALLYHO/Jng and C57BL/6J mice. A significant decrease was observed in the expression of nuclear- and mitochondrial-encoded subunits of complex I and IV, respectively, in TALLYHO/Jng mice, which coincided with significant reductions in their enzymatic activities. Furthermore, sequence variants were identified in oxidative phosphorylation complex subunits, a mitochondrial tRNA synthetase, and mitochondrial ribosomal proteins. Our data suggested that the lower expression and activity of oxidative phosphorylation complexes results in the diminished energy metabolism observed in TALLYHO/Jng mice. Sequence variants identified in mitochondrial proteins accentuated a defect in mitochondrial protein synthesis which also contributes to impaired biogenesis and oxidative phosphorylation in TALLYHO/Jng mice. These results demonstrated that the identification of factors contributing to mitochondrial dysfunction will allow us to improve the disease prognosis and treatment of obesity and type 2 diabetes in humans.

**Highlights**

Mitochondrial oxidative phosphorylation is impaired in TALLYHO/Jng mice.

Expression and activities of complex I and IV are diminished in TALLYHO/Jng mice.

Impaired mitochondrial protein synthesis is one of the contributing factors.

Mitochondrial sequence variants may contribute to mitochondrial biogenesis defects.

## 3.1. Introduction

Diabetes and obesity are two of the major metabolic diseases worldwide that are becoming an epidemic due to an increase in sedentary lifestyles. Type 2 diabetes (T2D) accounts for at least 90% of the diabetic cases and is commonly associated with an increased risk for morbidity and mortality (Bouret, Levin, & Ozanne, 2015; Romao & Roth, 2008). The etiology of obesity and T2D involves complex interactions of several susceptibility genes and environmental factors, making disease prevention difficult (Bouret et al., 2015; Romao & Roth, 2008). Animal models with both physiologic and genetic similarities to humans have become valuable resources for obesity and T2D studies; however, to date, only a few polygenic animal models exist (Rees & Alcolado, 2005). Recently, TALLYHO/Jng (TH) mice were developed as models for human obesity and type 2 diabetes (J. H. Kim et al., 2001). The polygenic pattern of inheritance in TH mice closely resembles the genetic inheritance in humans, making these mice ideal models for the identification of underlying molecular defects in diabetes and obesity related abnormalities (Grarup, Sandholt, Hansen, & Pedersen, 2014; J. H. Kim & Saxton, 2012).

TH mice encompass many aspects of the human T2D condition and are characterized by obesity, glucose intolerance, insulin resistance, hyperinsulinemia, hyperlipidemia, and hyperglycemia (in male mice) (J. H. Kim et al., 2006; J. H. Kim et al., 2005). Additionally, these

mice are also accompanied by lower energy expenditure and locomotor activity (X. Mao et al., 2014). Previous studies have suggested that the diet is an important modulator of susceptibility to obesity and T2D when TH mice are fed a high fat diet (Parkman et al., 2016). Interestingly, the diabetic condition was ameliorated and metabolic flexibility was significantly improved in TH mice treated with Bezafibrate (BEZ), a pan PPAR (peroxisome proliferator-activated receptor) activator (Franko et al., 2017). Several mapping studies were performed to identify significant quantitative trait loci (QTLs) that are linked to the obesity, diabetes, and hyperlipidemia phenotypes present in TH mice (J. H. Kim et al., 2005; Parkman et al., 2017; Stewart, Kim, Saxton, & Kim, 2010). Additionally, whole genome sequencing was also performed to identify causal variants of underlying metabolic diseases found in this animal model (Denvir et al., 2016).

Mitochondrial dysfunction, including impaired mitochondrial biogenesis, was established as a contributing factor to insulin resistance in obesity and T2D (Kelley et al., 2002; Patti et al., 2003; Petersen et al., 2003). Mitochondrial biogenesis relies on the coordinated regulation of both nuclear and mitochondrial protein expression for energy metabolism via oxidative phosphorylation (OXPHOS). In mammals, mitochondrial biogenesis is regulated by PPAR coactivator-1 alpha (PGC-1α), a transcriptional coactivator that interacts with nuclear respiratory factor 1 (NRF-1) and stimulates the transcription of nuclear-encoded mitochondrial genes (Heilbronn et al., 2007; Mootha et al., 2003; Patti et al., 2003). A coordinated reduction of PGC-1α and its responsive gene, NRF-1, were correlated with obesity, insulin resistance, and T2D (Heilbronn et al., 2007; Mootha et al., 2003; Patti et al., 2003; Skov et al., 2007). Lower expression of each of the five mitochondrial OXPHOS complexes, including the mitochondrial-encoded subunit of complex IV, cytochrome c oxidase I (COI), was shown in subjects with acquired obesity and insulin resistance (Heinonen et al., 2015; Morino et al., 2005; Perks et al.,

2017). Interestingly, one of the most common pathogenic mutations in the mitochondrial genome that is linked to T2D was found in the mitochondrial-encoded tRNA (Leu, UUR), which led to a defect in oxidative phosphorylation (Esterhuizen et al., 2019), and the mutation was identified in patients with impaired insulin secretion (Maassen et al., 2004; van den Ouweland et al., 1992).

The mitochondrial genome codes for 13 polypeptides that are essential subunits of OXPHOS complexes, two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). These essential subunits are synthesized by the mitochondrial transcription and translation machinery, which also contribute to the impaired mitochondrial biogenesis identified in obesity and T2D. Studies have demonstrated that the haploinsufficiency of pentatricopeptide repeat domain 1 (PTCD1) and the altered expression and/or mutation of mitochondrial ribosomal proteins (MRPs) led to reduced mitochondrial energy metabolism via decreased RNA processing, impaired mitochondrial protein synthesis, and the diminished expression of OXPHOS complexes in obesity, insulin resistance, and T2D (Bains et al., 2004; Heinonen et al., 2015; Perks et al., 2017; Rong et al., 2007). Additionally, the mitochondrial elongation factor Tu (EF-Tu) was also identified as a possible factor responsible for impaired mitochondrial biogenesis following exercise in an obese state (Greene et al., 2014) and has been recognized as one of the mitochondrial genes most connected to the insulin signaling cascade (Mercader et al., 2012). Taken together, these studies suggest that impaired mitochondrial biogenesis, including diminished mitochondrial protein synthesis, may lead to the metabolic disturbances that are characteristic of obesity, insulin resistance, and T2D. However, we still lack a thorough understanding of mitochondrial biogenesis and the contribution of mitochondrial protein synthesis and oxidative phosphorylation in obesity and T2D.

In this study, we investigated mitochondrial biogenesis, including the expression of nuclear- and mitochondrial-encoded subunits of OXPHOS complexes and mitochondrial translation machinery, in mitochondrial rich liver and kidney tissues of TH and C57BL/6J (B6) mice. We discovered reduced expression of nuclear-encoded subunits of OXPHOS complex I, NADH:ubiquinone oxidoreductase subunit B8 (NDUFB8) and NADH:ubiquinone oxidoreductase core subunit S2 (NDUFS2), and mitochondrial-encoded subunits of OXPHOS complex IV, COI and cytochrome c oxidase II (COII), along with diminished activities of the corresponding complexes. Furthermore, we identified sequence variants in NDUFS2, mitochondrial aspartyl-tRNA synthetase 2 (DARS2), and several MRPs in the whole genome sequence database of TH mice previously published (Denvir et al., 2016). The potential contribution of these sequence variants on mitochondrial biogenesis and oxidative phosphorylation is also discussed. Our study strongly indicates that impaired mitochondrial biogenesis could be one of the reasons for the lower mitochondrial oxidative energy metabolism in TH mice.

## 3.2. Materials and Methods

### 3.2.1. TALLYHO/Jng and C57BL/6J Tissue Samples

Liver (18-20 weeks old) and kidney (25-27 weeks old) tissues were collected from male TH and B6 mice fed standard rodent chow (Purina 5001, PMI Nutrition, Brentwood, MO, USA). Breeding of TALLYHO/Jng mice was established as previously described (J. H. Kim & Saxton, 2012; X. Mao et al., 2014) and all animal studies were carried out with the approval of Marshall University Animal Care and Use Committee.

### 3.2.2. Western Blot Analyses

Tissue lysates from the liver of TH and B6 mice (n=5 in each group) and the kidney of TH (n=3) and B6 (n=5) mice were obtained by sonication of samples in RIPA (radioimmunoprecipitation assay) buffer containing 50 mM Tris-HCl (Tris-hydrochloride) (pH 7.6), 150 mM NaCl (sodium chloride), 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 1% NP40 (nonylphenoxypolyethoxylethanol), 0.1% SDS (sodium dodecyl sulfate), 0.5% DOC (sodium deoxycholate), 1 mM PMSF (phenylmethylsulfonyl fluoride), and protease and phosphatase inhibitor cocktails (Abcam, Cambridge, MA). Protein concentration of tissue lysates was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, USA). Protein lysates were separated by 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and the gels were stained with Coomassie brilliant blue to ensure equal protein loading. The lysates (5 – 40 µg) were then separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad, Richmond, VA), stained with Ponceau S to check for equal protein loading, and blocked with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST), 5% (w/v) dry skim milk powder, and 1% BSA (bovine serum albumin). Membranes were incubated with primary antibodies overnight against the following proteins: OXPHOS cocktail from Mitosciences (Eugene, OR); ATP6 and COII from Abcam (Cambridge, MA); COI, NDUFS2, NRF-1, TFAM, and citrate synthase from Santa Cruz (Dallas, TX); SDHA from Novus Biologicals (Littleton, CO); GAPDH (glyceraldehyde 3-phosphate dehydrogenase) from Fitzgerald Industries International Inc. (Acton, MA); PDH, PEPCK, and PDHK1 from Cell Signaling Technology (Danvers, MA); MRPS29 and HSP60 from BD Biosciences (San Jose, CA); MRPL11 and MRPS18-B from Sigma Aldrich (St. Louis, MO); DARS2 from Proteintech

(Rosemont, IL); and MRPL15 and EF-Tu were a kind gift from Dr. Linda Spremulli. The protein immunoreactivity was detected using the ECL chemiluminescent kit from Amersham (GE Healthcare Life Sciences, Marlborough, MA), and the membranes were developed per the manufacturer's protocol. UN-Scan-It (Silk Scientific, Inc, Orem, UT) and ImageJ (Schneider, Rasband, & Eliceiri, 2012) were used to quantify the protein band intensities detected by Western blot analyses. The quantified values were normalized to the protein loading determined by Ponceau S staining and GAPDH probing of each membrane. The expression of each protein was quantified from each mouse sample from at least three independent experiments. The data are the mean ± SD for each mouse strain, and the results are expressed as a percentage of B6 mice.

### 3.2.3. Mitochondrial Complex Activity Assays

Protein lysates used in the activity assays for mitochondrial OXPHOS complexes were prepared in a buffer containing 10 mM Tris-HCl (pH 7.0), 250 mM sucrose, and phosphatase and protease inhibitor cocktails. For complex I and III enzymatic activity assays, liver and kidney protein lysates were further diluted in a 50 mM potassium phosphate (pH 7.5) buffer containing 1 mg/mL BSA, 1 mM potassium cyanide, and 100 µM cytochrome c. The protein concentration was determined by using the BCA protein assay and protein amounts were normalized with Coomassie brilliant blue stained gels. The reactions were started by adding 200 µM NADH and the combined complex I and III assay was determined spectrometrically by measuring the reduction of cytochrome c at 550 nm for 6 min. Each sample was run in triplicate (technical replicates) and each assay was run at least three times (biological replicates). The average absorbance values were calculated for both technical and biological replicates and were graphed against the time points to determine the linear range of the assay. The rate of cytochrome c

reduction was then calculated by a time-dependent change in the absorbance at the selected time point for each TH and B6 sample using the formula: Rate = (Abs 1 – Abs 2) / (Time 1 – Time 2). The data are the mean ± SD for each mouse strain and the results are presented as a percentage of B6 mice.

Briefly, liver and kidney lysates for the complex IV enzymatic activity assay were further diluted in a 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 100 µM reduced cytochrome c. The complex IV activity was also measured spectrometrically by monitoring the oxidation of cytochrome c at 550 nm, as previously described (Birch-Machin & Turnbull, 2001). The rate of cytochrome c oxidation was calculated as described above for the complex I and III assay.

### 3.2.4. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from liver and kidney tissues in the presence of TRIzol (Invitrogen, Carlsbad, CA) and converted to cDNA with the high capacity cDNA reverse transcription kit using random primers (Applied Biosystems, Inc., Foster City, CA). Quantitative real-time PCR (qRT-PCR) was carried out using the PowerUp SYBR green master mix (Applied Biosystems, Inc.), and samples were run on an Applied Biosystems Step One Plus instrument. The relative expression values were calculated using the ΔΔCt method for both biological and technical replicates (Schmittgen & Livak, 2008). The transcript expression values were normalized to GAPDH as the control. Results were presented as a percentage of B6 mice. The following sequences were used as primers: 16S rRNA forward 5’-TGAACGGCTAAACGAGGGTC-3’ and reverse 5’-TATTCTCCGAGGTCACCCCAA-3’; ND6 forward 5’-TATATTGCCGCTACCCCAATCC-3’ and reverse 5’-ATCCAGAGACTTGGGGATCT-3’; Cyt b forward 5’-TGCATACGCCATTCTACGCT-3’ and

reverse 5'-TGGGTGTTCTACTGGTTGGC-3'; COI forward 5'-TCGGAGCCCCAGATATAGCA-3' and reverse 5'-TTTCCGGCTAGAGGTGGGTA-3'; ATP6 forward 5'-ATCCACACACCAAAAGGACGA-3' and reverse 5'-GGAAGTGGGCAAGTGAGCTT-3'; and GAPDH forward 5'-GGTGAAGGTCGGTGTGAACG-3'and reverse 5'-CTCGCTCCTGGAAGATGGTG-3'.

#### 3.2.5. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.07. Statistically significant differences between TH and B6 mice were detected using unpaired Student's *t*-tests (2-tailed). All data are expressed as the mean ± SD (standard deviation), unless otherwise stated.

### 3.3. Results

#### 3.3.1. Mitochondrial Oxidative Phosphorylation is Impaired in TH Mice

Mitochondrial dysfunction was recognized as one of the key underlying factors in the development of T2D and identified as a contributing factor to T2D-dependent liver and renal failure (de Boer et al., 2011; El-Serag, Tran, & Everhart, 2004). In the initial characterization studies of TH mice, altered energy balance was reported (X. Mao et al., 2014). Furthermore, data mining of the whole genome sequencing analysis revealed the presence of several missense variants in the genes of nuclear-encoded OXPHOS complexes I and II, *Ndufs2* and *Sdhc*, respectively (Parkman et al., 2017). To determine changes in mitochondrial energy metabolism in this mouse model, we compared the expression and activity of mitochondrial OXPHOS complexes in the liver (Figs. 7 and 8) and kidney tissues (Figs. 12 and 13) of TH and B6 mice due to the high energy demands in these organs. We first evaluated the steady-state expression of OXPHOS complexes by Western blot analyses using antibodies raised against several subunits at varying protein amounts to determine the optimal amount for the consistent detection of

OXPHOS subunits in the liver (Fig. 7A) and kidney (Fig. 12A) of several tissues from both mouse strains. In the liver samples initially analyzed, the expression of nuclear-encoded subunits of complex I, NDUFB8 and NDUFS2, and the mitochondrial-encoded subunits of complex IV, COI and COII, was significantly lower at varying protein amounts in TH mice (Figs. 7A and 9). The consistent detection of diminished OXPHOS subunit expression was observed at 15 μg (Fig. 7A) with significantly decreased expressions of NDUFB8, NDUFS2, COI, and COII by approximately 55%, 40%, 60%, and 30%, respectively (Fig. 7A, bottom panel). Therefore, the remainder of the liver samples were run at this optimal protein amount. Similar to Fig. 7A, significant reductions were observed in the expression of NDUFB8 and NDUFS2 by approximately 40% and 35%, respectively, in the liver of TH mice (Fig. 7B, $P = 0.0002$ and $P = 0.0001$). The mitochondrial-encoded subunits of complex IV, COI and COII, were also decreased by 45% and 55%, respectively (Fig. 7B, $P = 0.001$). On the other hand, the expression of complex II subunits, SDHA and SDHB ($P = 0.4303$ and $P = 0.3027$), complex III subunit, UQCRC2 ($P = 0.1881$), and complex V subunits, ATP5A and ATP6 ($P = 0.1790$ and $P = 0.2233$), was comparable between the two mouse strains (Figs. 7B and 10A). The changes in expression of OXPHOS complex subunits were normalized to the Ponceau S staining and GAPDH probing of the membranes (Figs. 7 and 10B). To determine the effect of the impaired NDUFB8, NDUFS2, COI, and COII expressions on oxidative phosphorylation activity and energy metabolism, we performed complex I and III and complex IV enzymatic activity assays. As shown in Fig. 8A, the complex I and III enzymatic activity was significantly decreased by about 42% ($P = 0.0001$) in TH mice. Furthermore, a reduction of 25% ($P = 0.0003$) was clearly observed in the activity of complex IV (Fig. 8B). An example of the calculation used to determine the difference in enzymatic activities of OXPHOS complexes is described in the

Materials and Methods and demonstrated in Fig. 11. The diminished activity of OXPHOS complexes I, III, and IV coincided with the significantly lower expression of the NDUFB8, NDUFS2, COI, and COII subunits in the TH mice liver.

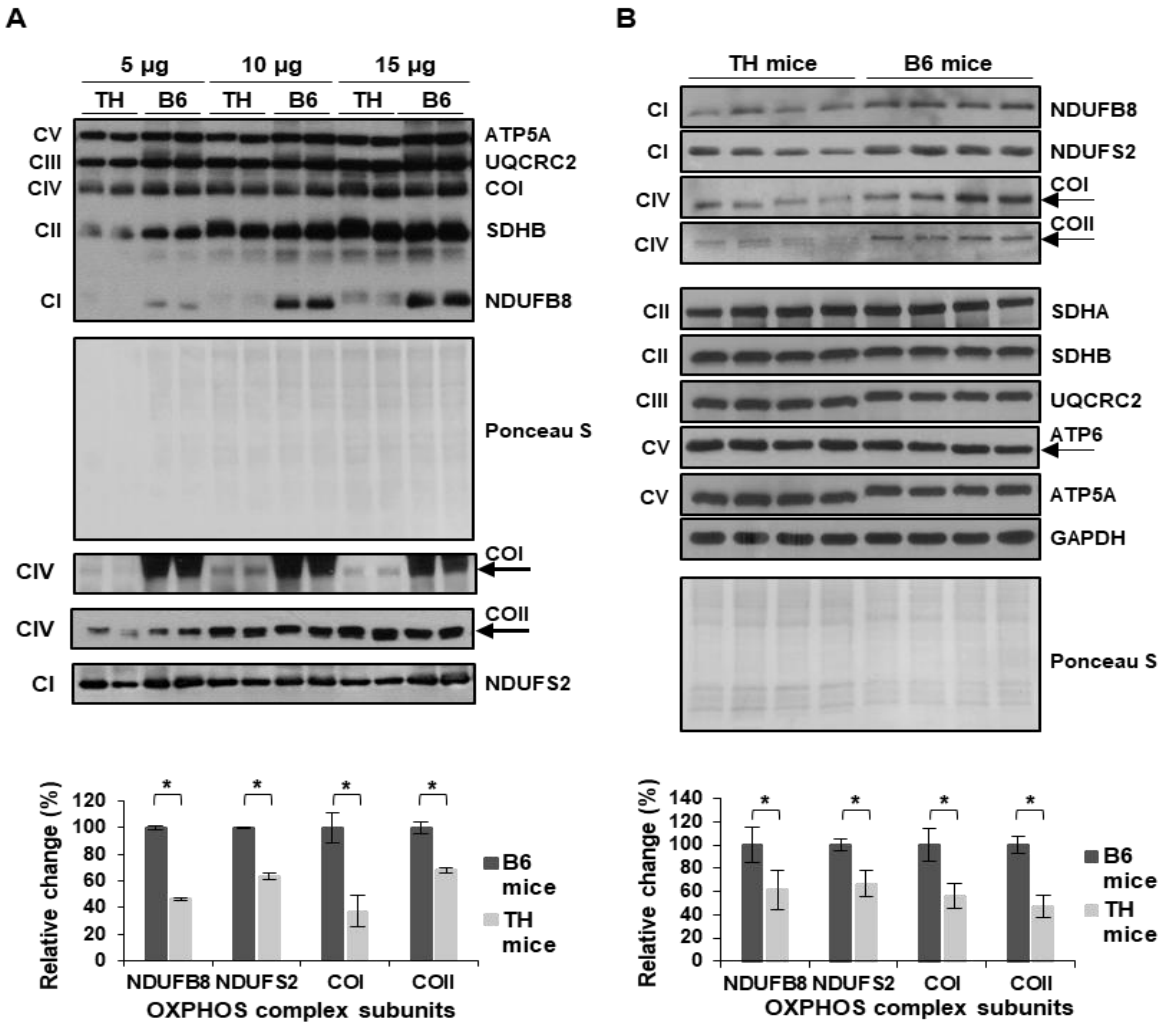



**Figure 7. Mitochondrial OXPHOS is impaired in TH mice liver**. **A)** To determine the linear response range of the antibodies, the expression of OXPHOS subunits NDUFB8 and NDUFS2 (complex I), SDHA and SDHB (complex II), UQCRC2 (complex III), COI and COII (complex IV), and ATP5A and ATP6 (complex V) was detected at varying protein amounts by Western blot analyses of TH and B6 mice liver lysates (n=2 in each group). The average quantitative analyses of the protein bands (15 µg) by densitometry are shown in the bottom panel. **B)** The expression of OXPHOS complexes in TH and B6 mice liver (n=4 in each group). The graph (bottom panel) represents the average quantitative analyses of the OXPHOS complex subunits with significant changes from each mouse strain at the optimal protein amount determined in A. The mitochondrial-encoded subunits COI, COII, and ATP6 are marked by arrows. Approximately 5 – 30 µg of protein lysate was separated by 12% SDS-PAGE and equal protein loading was evaluated by Ponceau S staining and GAPDH probing of the membranes. Results represent the mean ± SD of at least three experiments for each mouse strain and are presented as a percentage of B6 mice. Significant difference was observed between B6 and TH mice using an unpaired Student's *t*-test (2-tailed), *P < 0.05.

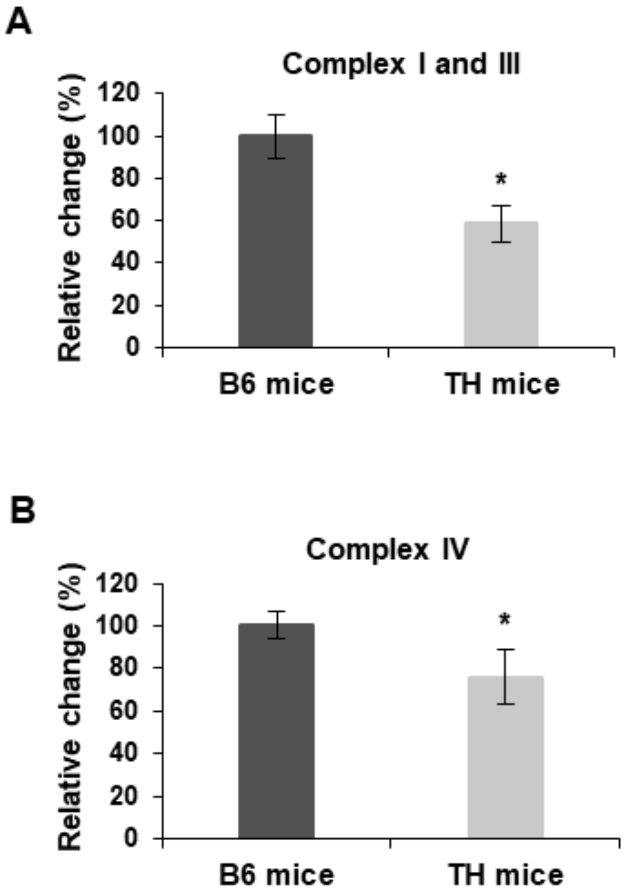



**Figure 8. Mitochondrial complex I and III and IV activities are lower in TH mice liver**. **A)** Combined complex I and III activity was determined by measuring cytochrome c reduction spectrometrically at 550 nm using equal amounts (~10 µg) of TH and B6 mice liver lysates. **B)** The rate of cytochrome c oxidation at 550 nm was determined as the complex IV activity using equal amounts (~20 µg) of liver lysates for both mouse strains. The data are the mean ± SD of at least three experiments for both mouse strains. Results are represented as a percentage of B6 mice. Significant difference was observed between B6 and TH mice using unpaired Student's *t*-tests (2-tailed), *P < 0.05.

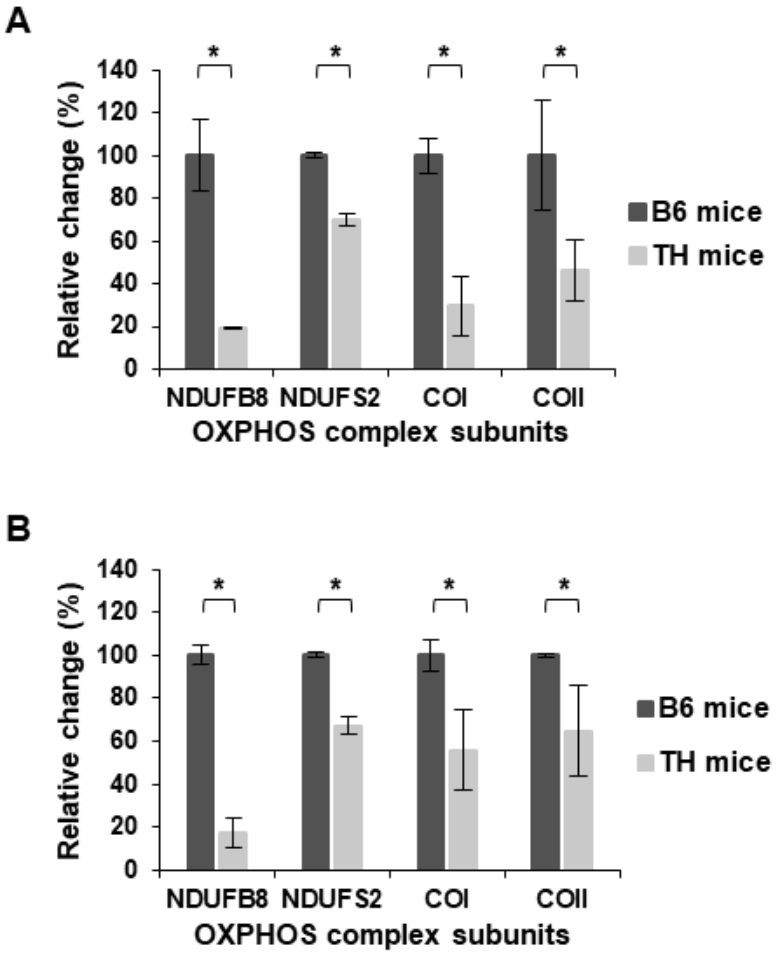



**Figure 9. Expression of mitochondrial OXPHOS complexes I and IV is diminished at various protein concentrations in the liver of TH mice**. **A)** Quantitation of OXPHOS complex subunits NDUFB8 and NDUFS2 (complex I) and COI and COII (complex IV) at 5 µg and 10 µg **(B)** in the liver of TH and B6 mice, Western blots shown in Fig. 7A. Results are represented as mean ± SD of at least three experiments and presented as a percentage of B6 mice. Statistical analyses were performed using the unpaired Student's *t*-test (2-tailed), *P < 0.05. See Figure 7 legend for details.

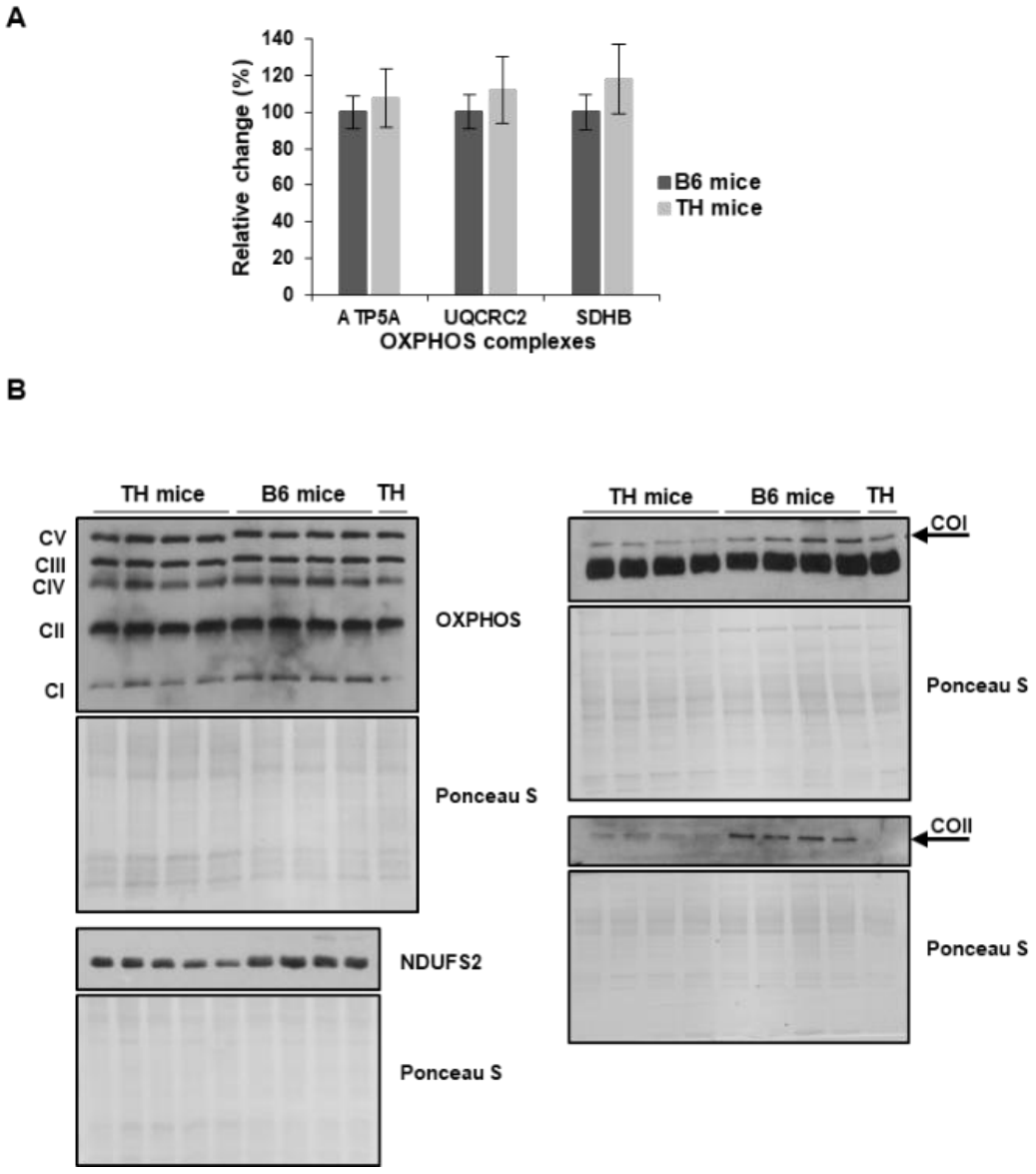



**Figure 10. Mitochondrial OXPHOS complex expression in the liver of TH mice**. **A)** Quantitation of OXPHOS complex expression in the liver of TH and B6 mice, shown in Fig. 7B. Results are represented as the mean ± SD of at least three experiments and presented as a percentage of B6 mice. The unpaired Student's *t*-test (2-tailed) was performed for statistical analysis, and no significant difference was observed in these OXPHOS subunits between the two mouse strains. **B)** Whole Western blots and the corresponding Ponceau S membrane stains for antibodies that show significant changes in the expression between TH and B6 mice. Protein bands for COI and COII are indicated by arrows. See Figure 7 legend for details.

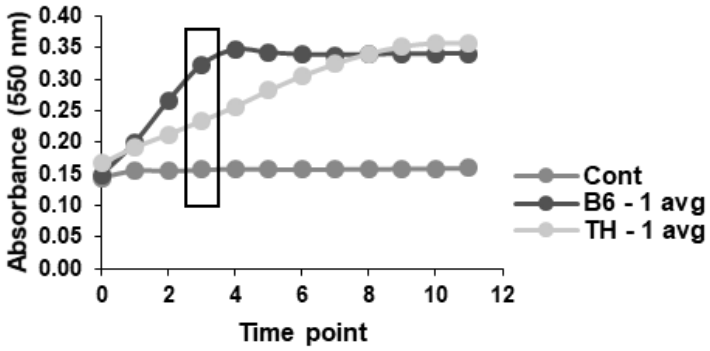
**A**

| | Cont | TH - 1 | TH - 1 | TH - 1 avg | | B6 - 1 | B6 - 1 | B6 - 1 avg |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| 0 | 0.144 | 0.168 | 0.167 | 0.167 | | 0.150 | 0.148 | 0.149 |
| 1 | 0.156 | 0.193 | 0.194 | 0.193 | | 0.203 | 0.197 | 0.200 |
| 2 | 0.155 | 0.212 | 0.214 | 0.213 | | 0.268 | 0.264 | 0.266 |
| 3 | **0.167** | **0.234** | **0.236** | 0.235 | | **0.322** | **0.324** | 0.323 |
| 4 | 0.159 | 0.256 | 0.258 | 0.257 | | 0.350 | 0.344 | 0.347 |
| 5 | 0.158 | 0.284 | 0.281 | 0.282 | | 0.346 | 0.338 | 0.342 |
| 6 | 0.157 | 0.308 | 0.303 | 0.305 | | 0.342 | 0.336 | 0.339 |
| 7 | 0.158 | 0.327 | 0.322 | 0.325 | | 0.341 | 0.336 | 0.339 |
| 8 | 0.158 | 0.342 | 0.339 | 0.340 | | 0.342 | 0.337 | 0.339 |
| 9 | 0.159 | 0.353 | 0.351 | 0.352 | | 0.343 | 0.337 | 0.340 |
| 10 | 0.159 | 0.358 | 0.356 | 0.357 | | 0.343 | 0.338 | 0.340 |
| 11 | 0.160 | 0.358 | 0.356 | 0.357 | | 0.344 | 0.338 | 0.341 |

(Abs 1 -Abs 2)/(Time 1- Time 2)

| | | | |
|---|---|---|---|
| | TH | TH | TH avg |
| $ | 0.066 | 0.068 | 0.067 |
| | B6 | B6 | B6 avg |
| | 0.172 | 0.176 | 0.174 |
| | | B6 | TH |
| | % change | 100.000 | 38.676 |
| | Std dev | 0.262 | 0.156 |

**B**




**C**

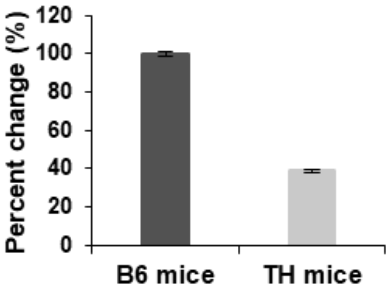



**Figure 11. Sample calculation for complex I and III enzymatic activity assays**. **A)** Absorbance values of cytochrome c reduction measured at 550 nm over six min. The values listed are from the control (without lysate), one TH, and one B6 mouse liver sample run two times each. The absorbance values for each mouse strain were averaged together and the percent difference was calculated. **B)** The plot represents the average TH and B6 absorbance values graphed against each time point. The time point used to calculate the percent difference is shown in the black box and bolded. **C)** The graph represents the mean ± SD of the percent change at the third (3) timepoint between the TH and B6 mice. This process was repeated for each TH and B6 mouse sample in both the liver and the kidney, and each assay was run at least three times. The samples were normalized to the control without lysate per assay. This normalization and calculations were performed for both the liver and kidney tissues on every mouse sample for both complex I and III and complex IV activity assays.

Similar to the results obtained with liver tissues, the biggest difference between TH and B6 mice kidney samples was observed in the expression of OXPHOS complexes I and IV (Figs. 12A and 12B). As shown in Figs. 12A and 14, the expression of NDUFB8, NDUFS2, COI, and COII were significantly lower at varying protein amounts in the TH kidney, with the optimal and consistent detection at 15 μg, similar to the liver lysates. Reductions of approximately 25% and 30% were detected in the expression of nuclear-encoded NDUFB8 and NDUFS2 subunits, respectively ($P = 0.0014$ and $P = 0.0009$), followed by a 40% decrease in the expression of mitochondrial-encoded complex IV subunits, COI and COII ($P = 0.001$ and $P = 0.0008$), in TH mice kidney (Figs. 12B). The expression of complex II and III subunits was comparable in the kidney of TH and B6 mice (Fig. 12B, $P = 0.2609$ and $P = 0.9736$ and Fig. 15A). The nuclear-encoded subunit of complex V, ATP5A, also had a similar expression between the two mouse strains ($P = 0.5741$); however, the expression of the mitochondrial-encoded subunit of complex V, ATP6, was slightly lower in the TH mice kidney samples although not significant (Fig. 12B, $P = 0.2464$ and Fig. 15A). The changes observed in the expression of OXPHOS complex subunits were normalized to both the Ponceau S membrane staining and the expression of GAPDH (Figs. 12 and 15B). We also performed OXPHOS complex activity assays to determine the effect of the lower expression of OXPHOS complexes I and IV on oxidative phosphorylation function in the kidney of these mice. Significant decreases of roughly 30% ($P = 0.0113$) and 27% ($P = 0.0005$) were observed in the activities of complexes I and III (Fig. 13A) and complex IV (Fig. 13B), respectively, in the TH mice compared to the B6 mice. These diminished activities of OXPHOS complexes were associated with the reduced expression of their subunits. Together, our findings show significant decreases in oxidative phosphorylation complex expression and

activity which implies mitochondrial energy metabolism is impaired in both the liver and the kidney of TH mice.

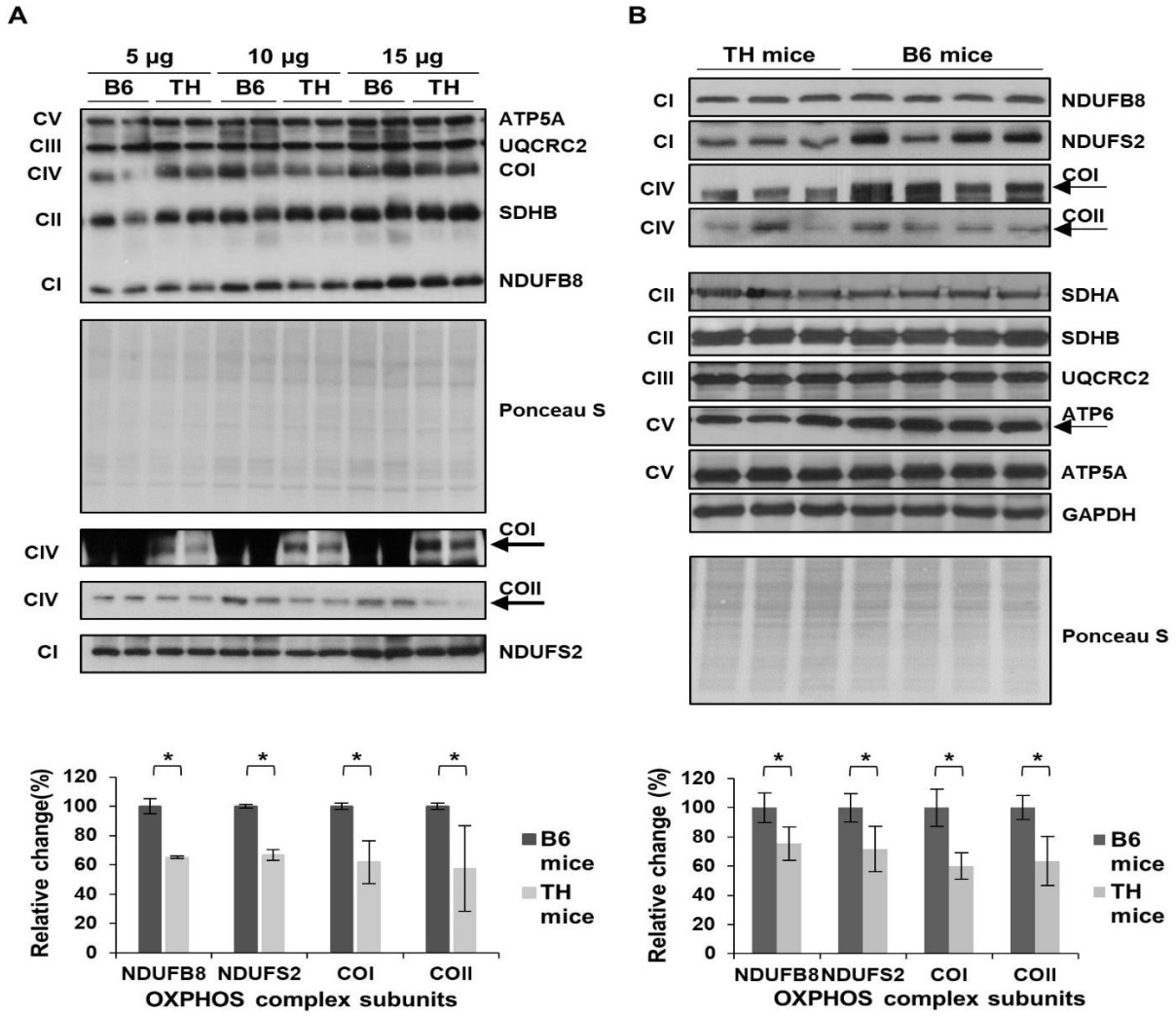



**Figure 12. Mitochondrial OXPHOS is reduced in TH mice kidney**. **A)** The expression of OXPHOS subunits in TH and B6 kidney tissue lysates (n=2 in each group) was evaluated at various protein amounts by Western blot analyses and Ponceau S staining. The average quantitative analyses by densitometry of OXPHOS subunits using 15 µg total protein lysate is shown in the bottom panel. **B)** Western blots of the OXPHOS subunit expression in TH (n=3) and B6 (n=4) mice kidney lysates. The graph in the bottom panel represents the average quantitative analysis of OXPHOS subunits with significant changes at the optimal protein amount determined in A. The expression of mitochondrial-encoded subunits COI, COII, and ATP6 are marked by arrows. Results represent the mean ± SD of at least three experiments for each mouse strain and are presented as a percentage of B6 mice. Significant difference was observed between the mouse strains using unpaired Student's *t*-tests (2-tailed), *P < 0.05. See Figure 7 legend for details.

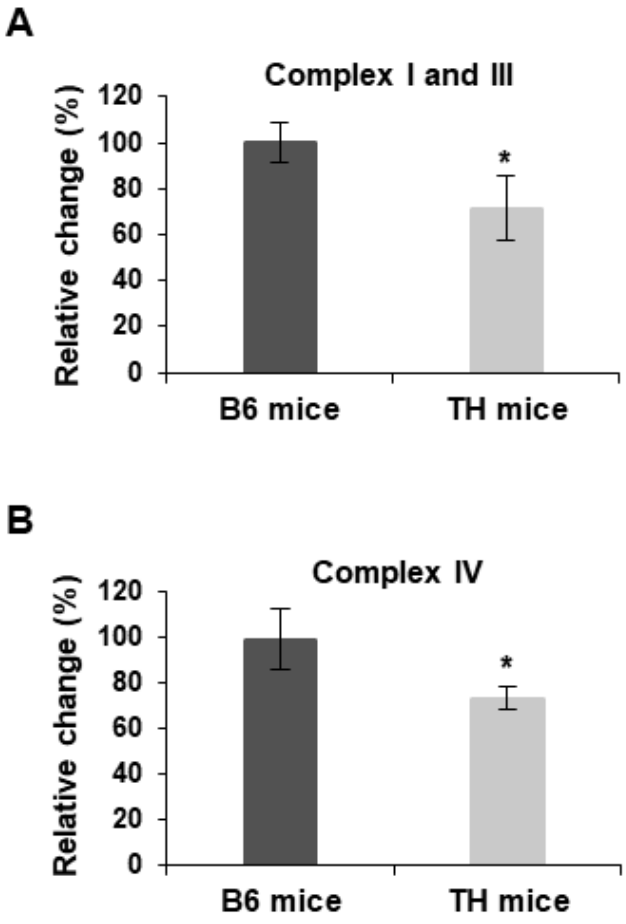



**Figure 13. Mitochondrial complex I and III and IV activities are diminished in the kidney of TH mice**. **A)** Combined complex I and III activity was determined by spectrometrically measuring the rate of cytochrome c reduction at 550 nm in TH and B6 mice kidney lysates. **B)** Complex IV activity was measured by the oxidation of cytochrome c at 550 nm in the kidney lysates. Results represent the mean ± SD of at least three experiments for each mouse strain and are expressed as a percentage of B6 mice. P-values were calculated using unpaired Student's *t*-tests (2-tailed), *P < 0.05. See Figure 8 legend for details.

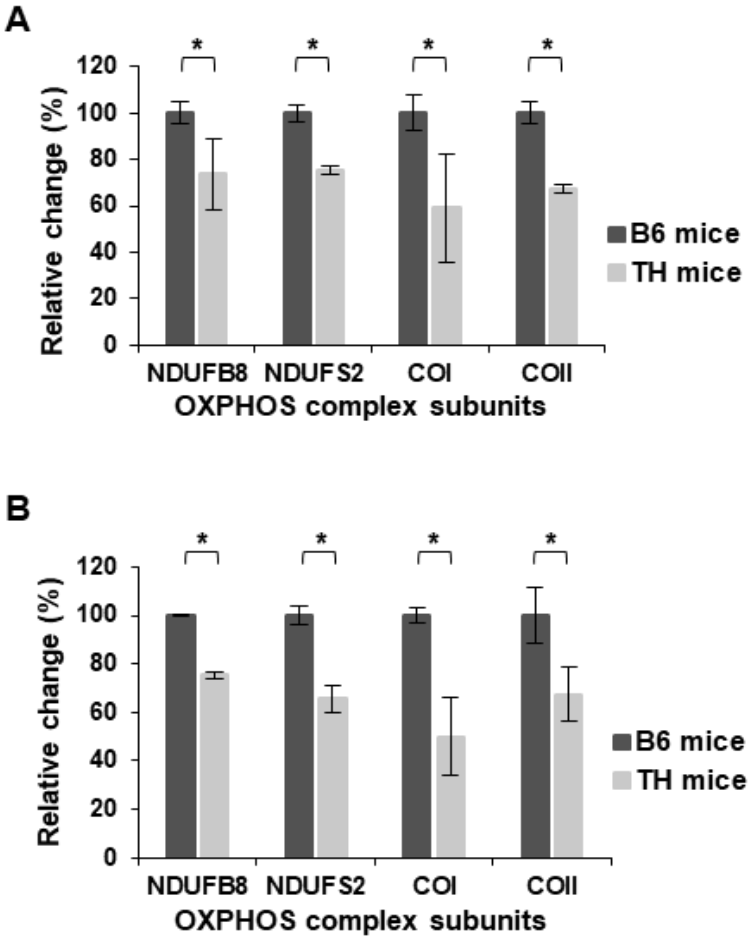



**Figure 14. Expression of mitochondrial OXPHOS complexes I and IV is diminished at various protein concentrations in TH mice kidney**. **A)** Quantitation of OXPHOS complex subunits of complex I, NDUFB8 and NDUFS2, and complex IV, COI and COII, at 5 µg and 10 µg **(B)** in the kidney of TH and B6 mice, Western blots shown in Fig. 12A. Results are represented as mean ± SD of at least three experiments and presented as a percentage of B6 mice. Statistical significance was measured by the unpaired Student's t-test (2-tailed), *P < 0.05. See Fig. 12 legend for details.

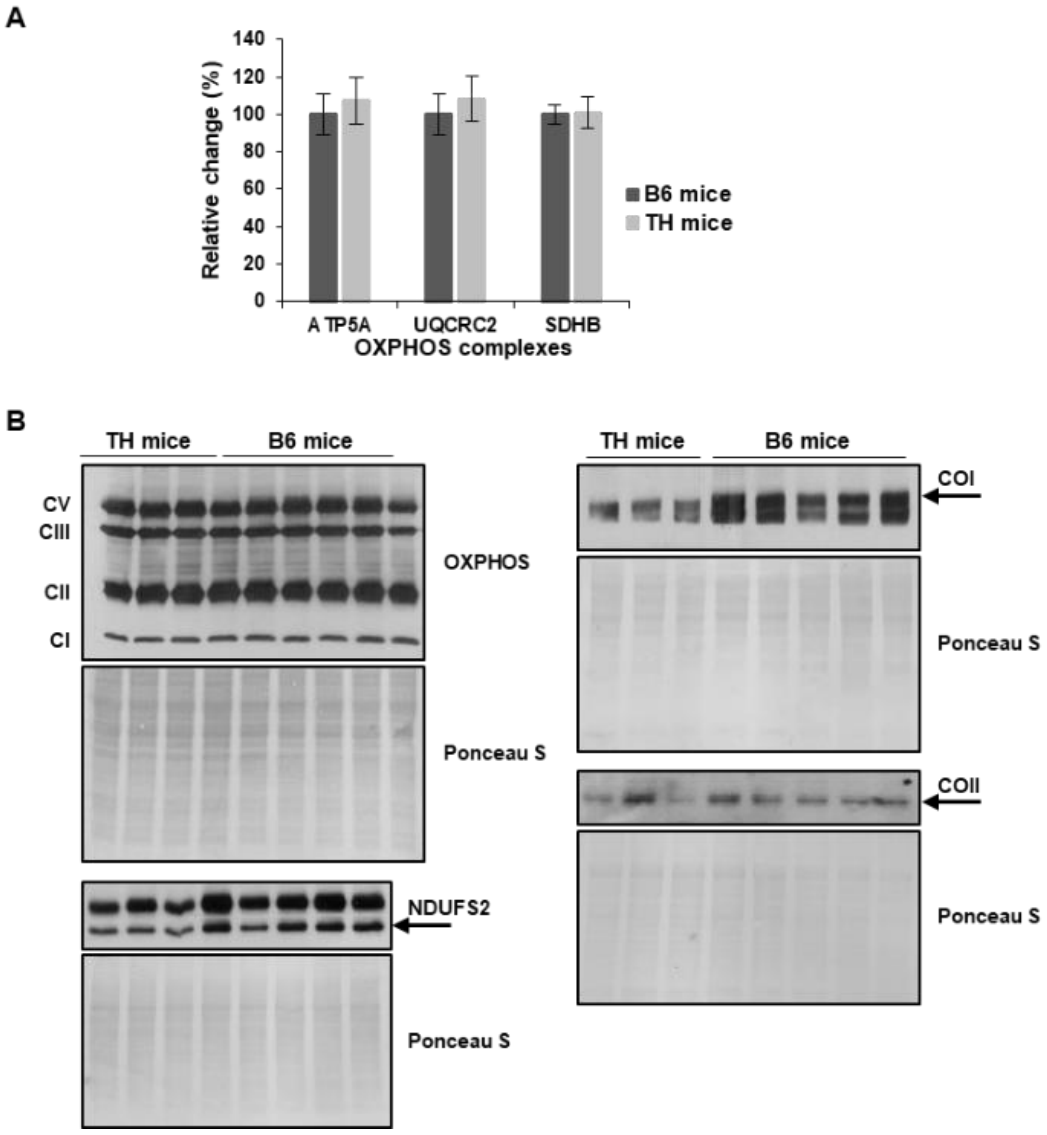



**Figure 15. Expression of mitochondrial OXPHOS complexes in the kidney of TH mice**. **A)** Quantitation of OXPHOS complex expression in the kidney of TH and B6 mice, shown in Fig. 12B. Results are represented as mean ± SD of at least three experiments and are presented as a percentage of B6 mice. The unpaired Student's *t*-test (2-tailed) was performed for statistical analysis, and no significant difference was observed in these OXPHOS subunits between the two mouse strains. **B)** The full Western blots and their corresponding Ponceau S stained membranes are shown for each OXPHOS subunit with significant changes in expression. The arrows indicate the COI, COII, and NDUFS2 protein bands for the TH and B6 mice kidney samples. See Fig. 12 legend for details.

### 3.3.2. Citrate Synthase Expression is Elevated in TH Mice

Above, we demonstrated that oxidative phosphorylation is significantly impaired in TH mice (Figs. 7-13). To further evaluate this decrease, we assessed the expression of several crucial metabolic enzymes that control OXPHOS by regulating the citric acid cycle (CAC) and gluconeogenesis, including citrate synthase (CS), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase 1 (PDHK1), and mitochondrial phosphoenolpyruvate carboxykinase (mtPEPCK). Interestingly, the expression of CS was increased by approximately 20-25% in the liver (Fig. 16A) and kidney (Fig. 16B) of TH mice. CS is one of the major checkpoints for the CAC and the increase in its expression suggests that the CAC activity is elevated in TH mice. Since CS expression is higher, we further analyzed other metabolic pathways that converge onto the CAC cycle. The expression of mtPEPCK, an enzyme that regulates the rate-controlling step of gluconeogenesis is comparable between the TH and B6 mice in the liver (Fig. 16A) but has a slight increase in expression in the kidney (Fig. 16B) of TH mice indicating that gluconeogenesis may be slightly higher. Little difference was observed in the expression of PDH and PDHK1, two critical enzymes involved in the regulation of pyruvate metabolism, in TH mice liver (Fig. 16A) and kidney (Fig. 16B) mitochondria. These findings imply that pyruvate metabolism and gluconeogenesis functions are comparable in both tissues of the two mouse strains and are not largely responsible for the increase in CS expression. Our results suggest that the lower oxidative metabolism in TH mice is not caused by diminished activities of other metabolic pathways that regulate OXPHOS complexes. Rather, the decrease is due to the reduced expression of OXPHOS complexes I and IV, possibly by a defect in nuclear and mitochondrial protein synthesis.

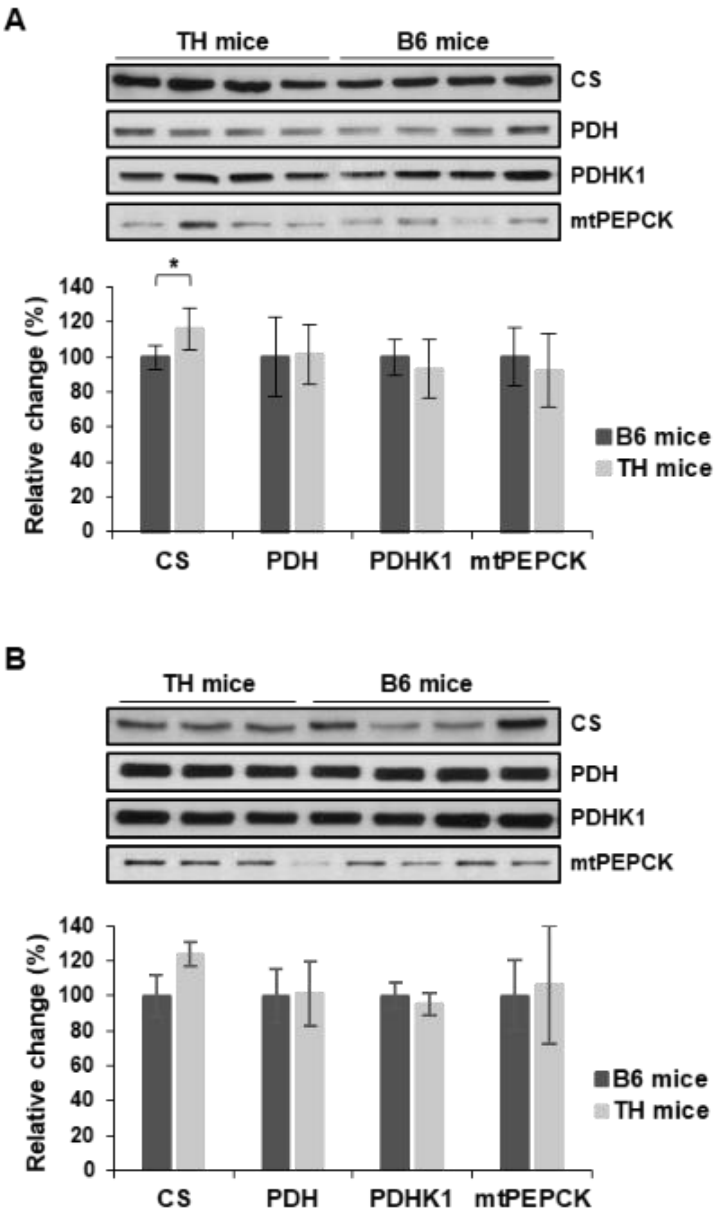



**Figure 16. Citrate synthase is increased in TH mice liver and kidney**. **A)** The expression of various crucial metabolic enzymes involved in the citric acid cycle and gluconeogenesis, including citrate synthase (CS), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase 1 (PDHK1), and mitochondrial phosphoenolpyruvate carboxykinase (mtPEPCK), was assessed in TH and B6 mice liver and kidney **(B)** by Western blot analyses. The quantitative analyses of metabolic enzymes by densitometry were normalized to the expression of GAPDH and Ponceau S stained membranes (bottom panels) as equal loading controls. Results are expressed as the mean ± SD for each mouse strain and presented as a percentage of B6 mice. Unpaired Student's *t*-tests (2-tailed) were performed to for statistical analyses and no significant difference was observed between TH and B6 mice.

### 3.3.3. Mitochondrial Transcription Machinery and Translation Components Remain Unaltered in TH mice

In mammals, OXPHOS complexes are encoded by approximately 85 nuclear and 13 mitochondrial genes. Mitochondrial biogenesis, mainly via transcription and translation of these genes, is essential for energy generation by oxidative phosphorylation. We observed a diminished protein expression of OXPHOS subunits NDUFB8, NDUFS2, COI, and COII in the liver and kidney of TH mice (Figs. 7 and 12). Since COI and COII are two of the 13 crucial mitochondrial-encoded subunits, we performed quantitative RT-PCR analyses on several mitochondrial-encoded transcripts to determine if a defect is present in the expression of mitochondrial genes in TH mice. Despite a lower protein expression of COI in the liver (Fig. 7) and kidney (Fig. 12) of TH mice, the mRNA expression of COI was comparable in both tissues between the two mouse strains (Figs. 17A and 18A). Furthermore, minor reductions were observed in the mRNA expression of mitochondrial-encoded 16S rRNA and OXPHOS subunits ND6 (complex I), cyt b (complex III), and ATP6 (complex V) between the TH and B6 mice liver (Fig. 19A) and kidney (Fig. 18A). To further evaluate this slight reduction, we performed Western blot analyses to assess the protein expression of two major transcription factors responsible for the synthesis of both mitochondrial- and nuclear-encoded proteins. The expression of mitochondrial transcription factor A (TFAM) and NRF-1 was comparable between TH and B6 mice in both the liver (Fig. 17B) and kidney (Fig. 18B). These results suggest that changes in the expression of mitochondrial transcription factors is not responsible for the slight reduction observed in the mitochondrial-encoded transcripts and further implies that there is no defect in the mitochondrial transcription of TH mice.

The minor differences found in the expression of mitochondrial-encoded rRNA/mRNA transcripts without changes in the expression of transcription factors, prompted us to analyze two essential mitochondrial translation factors, EF-Tu and DARS2, and ribosomal proteins that are responsible for the synthesis of the 13 mitochondrial-encoded proteins of the OXPHOS complexes in TH and B6 mice. Although EF-Tu has been associated with insulin signaling and the obesity phenotype (Greene et al., 2014; Mercader et al., 2012), the expression of EF-Tu in the obesity and insulin resistant TH mouse model is comparable to the B6 mice in both the liver (Fig. 17C) and kidney (Fig. 18C) tissues. In the whole genome sequencing analysis of TH mice, mitochondrial aspartyl-tRNA synthetase, DARS2, was shown to have a C370R mutation. Although this variant introduces a positively charged residue into the protein sequence in a highly conserved region, the difference in the expression of the mitochondrial DARS2, was negligible between the two mouse strains in both the liver and kidney (Figs. 17C and 18C, respectively). Interestingly, this C370R variant is also found in *Myotis lucifugus* (little brown bat) and *Equus caballus* (horse) sequences. Thus, the defect we observed in mitochondrial translation, indicated by the significantly diminished expression of mitochondrial-encoded COI and COII without changes in transcription, could not be exclusively dependent on the DARS2 variant.

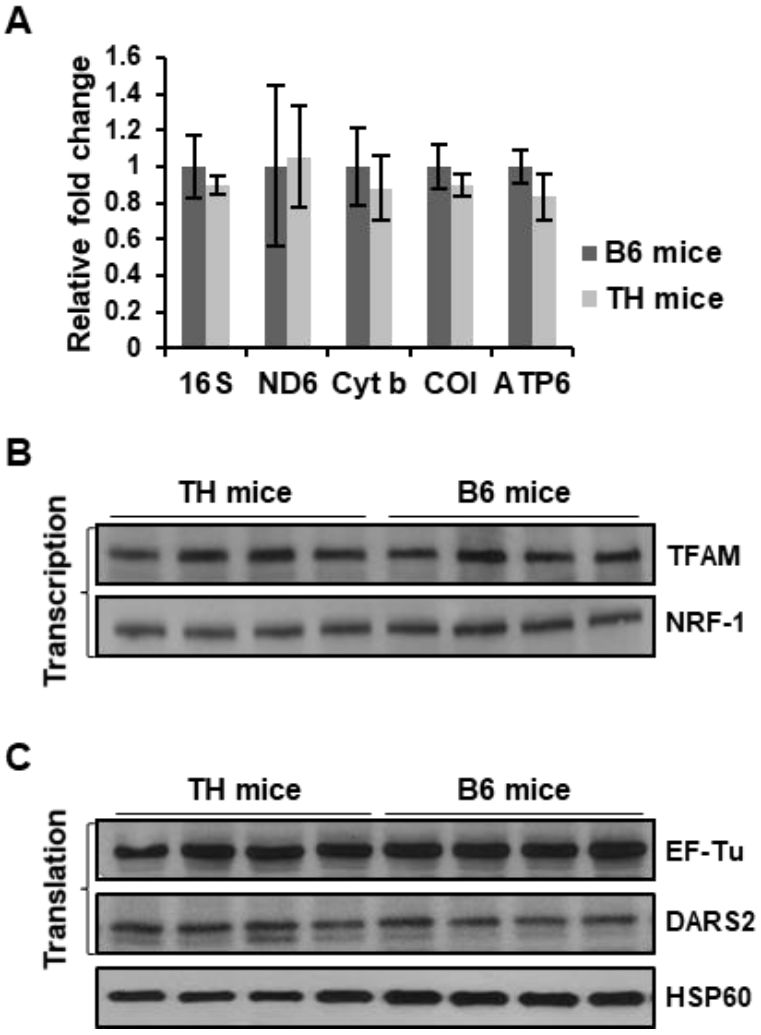



**Figure 17. Mitochondrial transcription and translation are comparable between TH and B6 mice liver**. **A)** Relative changes in mitochondrial transcripts, including 16S rRNA (39S subunit), ND6 (complex I), cyt b (complex III), COI (complex IV), and ATP6 (complex V) were determined by quantitative RT-PCR and reported as fold changes with respect to GAPDH mRNA expression in the liver of TH and B6 mice. Results represent the mean ± SD of at least three experiments for each mouse strain. Unpaired Student's *t*-tests (2-tailed) were performed for statistical analyses and no significant difference was shown between TH and B6 mice. **B)** Expression of mitochondrial transcription factors, TFAM and NRF-1, and translation factors, EF-Tu and DARS2 **(C)**, were evaluated in the liver of both mouse strains by Western blot analyses. Equal protein loading was determined by Ponceau S staining and probing membranes with mitochondrial matrix marker, HSP60.

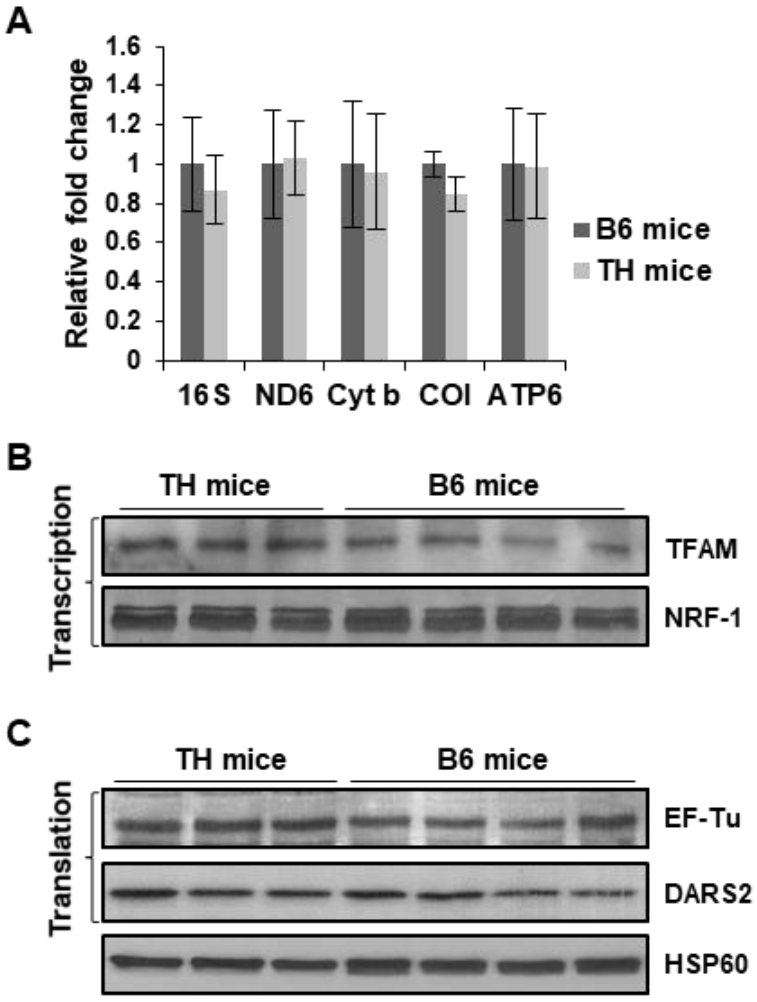



**Figure 18. Mitochondrial transcription and translation are unaltered in TH mice kidney**. **A)** Relative changes in 16S rRNA and mitochondrial-encoded mRNA expression were determined by quantitative RT-PCR in the kidney of TH and B6 mice and reported as fold changes with respect to GAPDH mRNA expression as a control. Results represent the mean ± SD of at least three experiments for each mouse strain. **B)** The expression of mitochondrial transcription and translation **(C)** factors were evaluated in TH and B6 mice kidney by Western blot analyses. See Figure 17 legend for details.

### 3.3.4. Expression of Mitochondrial Ribosomal Proteins are Comparable

Mitochondrial ribosomes, 55S, are composed of small (28S) and large (39S) subunits with two mitochondrial encoded rRNAs, 12S and 16S, respectively, and approximately 80 nuclear-encoded ribosomal proteins (E. C. Koc et al., 2013). These MRPs are essential to the synthesis of the 13 mitochondrial-encoded subunits of OXPHOS complexes in mammals. To identify changes in MRPs that could contribute to the impaired protein synthesis in TH mice, we analyzed several MRPs in both the 28S (MRPS18B and MRPS29) and 39S (MRPL11 and MRPL15) ribosomal subunits by Western blot analyses (Fig. 19). The expression of the majority of the MRPs analyzed, MRPS18B, MRPL11, and MRPL15, were comparable between the TH and B6 mice in both tissues (Figs. 19A and 19B) relative to GAPDH as a loading control. Surprisingly, the expression of MRPS29 was approximately 20% lower in the TH mice in both the liver (Fig. 19A) and kidney (Fig. 19B); however, the decrease was not statistically significant ($P = 0.0628$ and $P = 0.0558$).

The slight reduction observed in the expression of MRPS29 encouraged us to search for MRP variants in the whole genome sequence database previously published (Denvir et al., 2016), which is archived at the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) via accession number SRP067703. Our data mining revealed the presence of 16 sequence variants in 11 MRPs in TH mice (Table 3). To predict the amino acid substitution on MRP function, the ‘Sorting Intolerant From Tolerant’ (SIFT) scores were calculated (Table 3). The majority of the SIFT scores for the MRP sequence variants identified were greater than 0.05, indicating that the variants were tolerated (Zerbino et al., 2018). The majority of significant amino acid replacements were found at the N-terminal signal sequences of MRPs (Table 3). One of the most remarkable variants was identified in MRPL20. The missense sequence variant found

in MRPL20 caused the loss of a stop codon (Table 3), which introduces an additional nine amino acid residues into the protein sequence. The possible effect of this insertion at the C-terminus of MRPL20 is unknown. Although the sequence variants identified in mitochondrial ribosomal proteins in TH mice were possibly ineffective in altering the protein expression of other MRPs (Figs. 19A and 19B), the variants may still interfere with the translocation of MRPs into the mitochondria and the function of the ribosome.

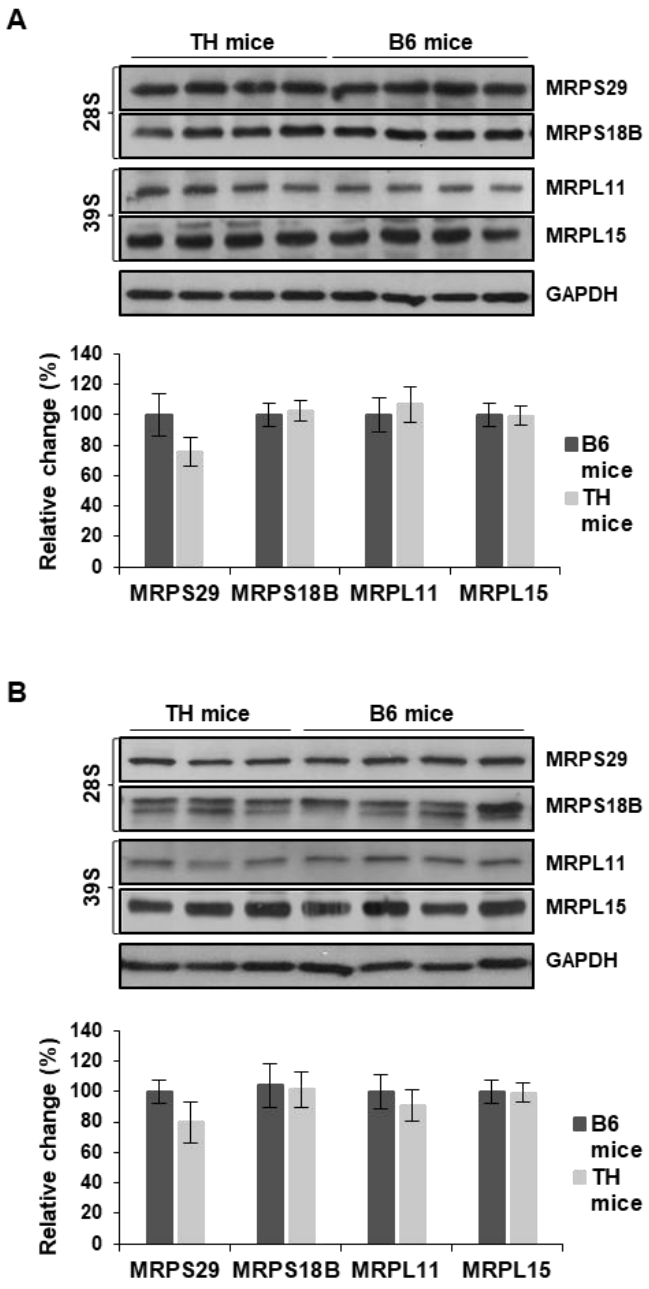



**Figure 19. Mitochondrial ribosomal protein expression remains unchanged in TH and B6 mice**. **A)** Relative expression of mitochondrial ribosomal proteins (MRPs) of the small (28S) and large (39S) subunits were detected in TH and B6 mice liver and kidney lysates **(B)** by Western blot analyses. The average quantitative analyses by densitometry of MRPs in TH and B6 mice normalized to GAPDH and Ponceau S staining is shown in the bottom panels. Results represent the mean ± SD of at least three experiments in each mouse strain and are presented as a percentage of B6 mice. No statistical difference was shown between TH and B6 mice using unpaired Student's *t*-tests (2-tailed). See Figure 7 legend for details.

| Gene | AA B6/TH | AA position | UniProtKB | SIFT score |
|---|---|---|---|---|
| **Mitochondrial ribosomal protein mutations** | | | | |
| **MRPL20** | **stop lost** | **97** | **Q9CQL4** | |
| | | | | |
| **MRPS11** | **R/L** | **13** | **Q9DCA2** | **0.12** |
| | **L/R** | **70** | | **0.06** |
| **MRPL22** | **A/T** | **4** | **Q8BU88** | **0.26** |
| **MRPL49** | **L/F** | **6** | **Q9CQ40** | **1.00** |
| **MRPL48** | **I/V** | **148** | **Q8JZS9** | **1.00** |
| **MRPS35** | **T/S** | **11** | **Q8BJZ4** | **0.37** |
| | **C/Y** | **216** | | **1.00** |
| | **I/V** | **293** | | **1.00** |
| **MRPL3** | **I/T** | **32** | **Q99N95** | **0.48** |
| **MRPL19** | **C/R** | **39** | **Q9D338** | **0.35** |
| | **E/Q** | **72** | | **0.20** |
| **MRPL39** | **K/R** | **133** | **Q9JKF7** | **0.49** |
| | **V/A** | **280** | | **0.52** |
| **MRPL55** | **L/F** | **19** | **Q9CZ83** | **0.65** |

**Table 3. Sequence variants of mitochondrial ribosomal proteins (MRPs) in TH mice** (Denvir et al., 2016).
AA: amino acid. Mitochondrial ribosomal small (MRPS) and (MRPL) subunit proteins. Sorting intolerant from tolerant (SIFT) scores are shown to predict if the amino acid substitution is deleterious (SIFT < 0.05) or tolerated/neutral (Zerbino et al., 2018).

### 3.4. Discussion

Obesity and T2D are two metabolic diseases that are becoming more prevalent worldwide. Recent evidence implies that mitochondrial dysfunction is an underlying defect in the development of obesity, insulin resistance, and T2D (Kelley et al., 2002; Patti et al., 2003; Petersen et al., 2003). The TH mouse has recently been developed as a polygenic model for obesity, insulin resistance, and T2D (J. H. Kim & Saxton, 2012; J. H. Kim et al., 2001). Metabolic characterization, gene mapping, and whole genome sequencing analyses of TH mice have revealed an altered energy balance (Franko et al., 2017; X. Mao et al., 2014; Parkman et al., 2017; Parkman et al., 2016); however, the mechanism is largely unknown. In this study, we evaluated mitochondrial biogenesis including the expression of mitochondrial- and nuclear-encoded OXPHOS complex subunits in mitochondrial rich tissues of TH and B6 mice. Additionally, we analyzed mitochondrial energy metabolism by observing changes in oxidative phosphorylation activity. In conjunction with the whole genome sequencing information, our results allowed us to correlate the biogenesis of OXPHOS complexes with mitochondrial translation, oxidative phosphorylation, and energy metabolism in TH mice (Fig. 20).

One of our most compelling observations was the significant reduction in the expression of nuclear-encoded complex I subunits, NDUFS2 and NDUFB8, and mitochondrial-encoded complex IV subunits, COI and COII, which coincided with diminished enzymatic activities of these OXPHOS complexes in TH mice liver and kidney tissues (Figs. 7-13). Strikingly, NDUFS2 carries an R8G variant which is located within the mitochondrial targeting sequence in TH mice. This variant can interfere with the translocation of NDUFS2 into the mitochondria. In fact, NDUFS2 is one of the largest and highly conserved subunits in complex I and its mutations have resulted in the disruption of the subunit as well as an absence in the formation of the

peripheral arm of complex I (Guenebaut, Vincentelli, Mills, Weiss, & Leonard, 1997). Complex I is the major entry point into the OXPHOS complexes and plays a pivotal role in mitochondrial energy metabolism. Several mutations found in complex I subunits and assembly factors were shown to cause a variety of clinical symptoms (Ugalde, Janssen, van den Heuvel, Smeitink, & Nijtmans, 2004). For example, mutations in *NDUFA4* caused a reduction in mitochondrial function, impaired insulin release in the pancreas, and led to the development of diabetes (Yagil, Varadi-Levi, & Yagil, 2018). The diminished expression of NDUFS2 could disrupt the subunit, inhibit the assembly of the peripheral arm of complex I, impair its function, significantly lower mitochondrial oxidative phosphorylation and energy metabolism, and contribute to the obesity and insulin resistant phenotypes observed in TH mice.

In addition to defects in complex I, the reduced expression of mitochondrial-encoded COI and COII and the diminished activity of complex IV were evident in TH mice. Interestingly, the expression of complex IV is required for the assembly and stability of complex I (Diaz, Fukui, Garcia, & Moraes, 2006); therefore, the diminished expression of complex IV subunits may contribute to the reduced expression of complex I observed in TH mice. Previous studies have also shown a lower expression of COI in obesity and insulin-resistant subjects (Heinonen et al., 2015; Morino et al., 2005; Perks et al., 2017). These results further implied that metabolic alterations could lead to the development of obesity-related diseases, such as T2D, and may be caused by impaired mitochondrial biogenesis and oxidative phosphorylation, as demonstrated in TH mice. Mitochondrial biogenesis relies on the coordinated expression of both nuclear- and mitochondrial-encoded OXPHOS subunits for energy metabolism. More significantly, complexes I and IV consist of 10 of the 13 mitochondrial-encoded essential subunits synthesized by the mitochondrial translation machinery. Thus, their expression is more susceptible to the

changes in mitochondrial protein synthesis. Although we observed minor differences in the expression of mitochondrial transcription and translation factors, the reduction in the expression of COI and COII subunits indicated that mitochondrial protein synthesis is impaired in TH mice. One of the possible candidates for the defect in mitochondrial translation was the DARS2 variant, C370R, identified in TH mice. The variant introduced a positive charge into a highly conserved region of DARS2 near the tRNA binding site, which can alter aminoacylation of $mttRNA^{Asp}$ and impair mitochondrial protein synthesis (Fig. 20). However, the presence of this variant in *M. lucifugus* and *E. caballus* DARS2 gene, demonstrated that the diminished mitochondrial protein synthesis observed in TH mice is not exclusively caused by the C370R conversion.

A negligible difference was observed in the expression of the majority of the MRPs analyzed, except a slightly lower expression of MRPS29 in TH mice. MRPS29 provides the only GTPase activity to the 28S subunit, making its expression essential for ribosome function and the regulation of mitochondrial protein synthesis (Cavdar Koc, Ranasinghe, et al., 2001; O'Brien, O'Brien, & Norman, 2005). Although the reduction observed in TH mice is not statistically significant, the decreased expression of MRPS29 can impair the ribosome assembly and function reducing protein synthesis and oxidative phosphorylation activity. The probability of defects in mitochondrial translation machinery compelled us to search for sequence variants in translation factors and MRPs in the whole genome sequence database of TH mice. The sequence variants found in 11 MRPs of TH mice were not predicted to be deleterious based on the SIFT scores (> 0.05) (Table 3). In fact, the majority of the variants were either at the less conserved N-terminus of the MRPs or the amino acid substitutions were conservative replacements with similar amino acid residues (Table 3). The variants at the N-terminus could interfere with the translocation of

MRPs into the mitochondria, which may impair the assembly and function of the mitochondrial ribosome and inhibit protein synthesis. Perhaps the most compelling variant found in TH mice was in MRPL20. The loss of the stop codon and the nine residue extension of MRPL20 may alter the folding, structure, and/or activity of the protein, in addition to its ability to interact with other MRPs in the mitochondrial ribosome. Moreover, these extra residues make MRPL20 a viable candidate for the defect found in mitochondrial protein synthesis contributing to impaired mitochondrial biogenesis and oxidative phosphorylation in TH mice.

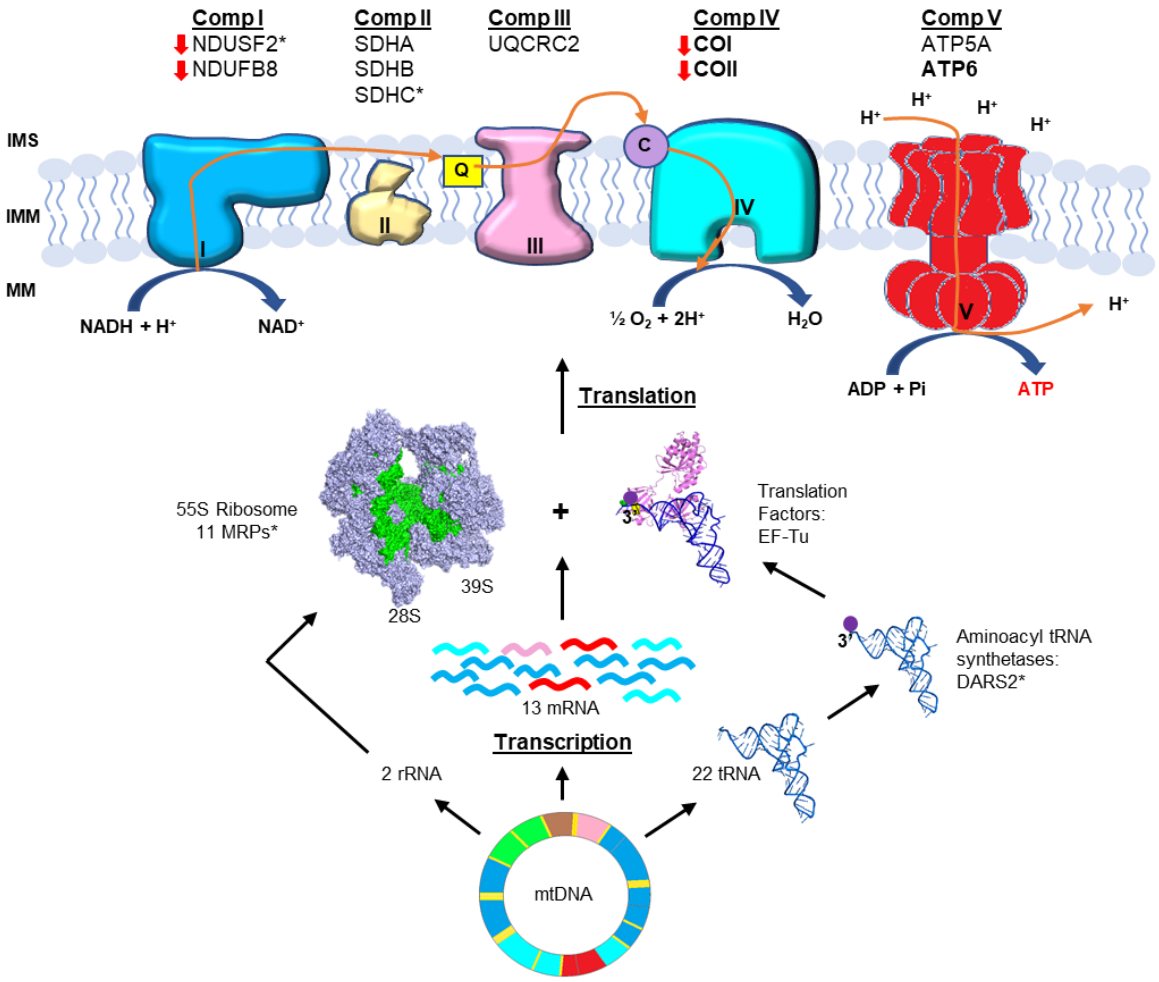



**Figure 20. A schematic synopsis of OXPHOS and mitochondrial translation component variants found in TH mice**. Mammalian mitochondria have a ~16.5 kb circular genome (mtDNA) that encodes two rRNAs, 22 tRNAs, and 13 mRNAs (nine monocistronic and two dicistronic). The mammalian mitochondrial 55S ribosome (28S and 39S subunits), are responsible for the synthesis of the 13 mitochondrial-encoded subunits of complexes I, III, IV, and ATP synthase (complex V). The expression of mitochondrial-encoded subunits COI, COII, and ATP6 detected in TH mice are bolded. The expression of nuclear-encoded mitochondrial OXPHOS complex subunits NDUFB8 and NDUFS2 (complex I), SDHA, SDHB, and SDHC (complex II), UQCRC2 (complex III), ATP5A (complex V) were also detected in TH mice. The expression of nuclear-encoded NDUFB8 and NDUFS2 and mitochondrial-encoded COI and COII were lower in TH mice, indicated by red arrows. Missense variants found in OXPHOS complexes, MRPs, and mitochondrial aspartyl-tRNA synthetase 2 (DARS2) in TH mice are shown by asterisks (*). Bovine mtEF-Tu (PDB #1XB2) and *E. coli* aminoacyl-tRNA$^{Cys}$ (PDB #IB23) were modeled to demonstrate the interaction of tRNA with mtEF-Tu and shown in violet and blue, respectively. The bovine 55S mammalian ribosome (PDB #3J9M) was also modeled and shown in light purple, with the mitochondrial rRNAs highlighted in green.

### 3.5. Conclusions

In summary, our data clearly demonstrated a significant impairment of mitochondrial energy metabolism, observed through a significant reduction in oxidative phosphorylation in TH mice. The sequence variants discussed in this study may be contributing factors to the diminished mitochondrial biogenesis by inhibiting protein synthesis in TH mice. Further studies need to be performed to determine the contribution of mitochondrial defects to the development of obesity, insulin resistance, and T2D in this animal model.

### 3.6. Conflict of Interests

The authors declare they have no conflict of interest.

### 3.7. Acknowledgements

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# CHAPTER 4

## C-SRC KINASE IMPAIRS THE EXPRESSION OF MITOCHONDRIAL OXPHOS COMPLEXES IN LIVER CANCER

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**Abstract**

Src family kinases (SFKs) play a crucial role in the regulation of multiple cellular pathways, including mitochondrial oxidative phosphorylation (OXPHOS). Aberrant activities of one of the most predominant SFKs, c-Src, was identified as a fundamental cause for dysfunctional cell signaling and implicated in cancer development and metastasis, especially in human hepatocellular carcinoma (HCC). However, the role of c-Src in mitochondrial energy metabolism in liver cancer remains to be elucidated. In this study, we investigated the effect of c-Src expression on mitochondrial energy metabolism by examining changes in the expression and activities of OXPHOS complexes in liver cancer biopsies and cell lines. An increased expression of c-Src was correlated with an impaired expression of nuclear- and mitochondrial-encoded subunits of OXPHOS complexes I and IV, respectively, in metastatic biopsies and cell lines. Additionally, we observed a similar association between high c-Src and reduced OXPHOS complex expression and activity in mouse embryonic fibroblast (MEF) cell lines. Interestingly, the inhibition of c-Src kinase activity with the SFK inhibitor PP2 and c-Src siRNA stimulated the expression of complex I and IV subunits and increased their enzymatic activities in both cancer and normal cells. Evidence provided in this study reveals that c-Src impairs the expression and function of mitochondrial OXPHOS complexes, resulting in a significant defect in mitochondrial energy metabolism, which can be a contributing factor to the development and progression of liver cancer. Furthermore, our findings strongly suggest that SFK inhibitors should be used in the treatment of HCC and other cancers with aberrant c-Src kinase activity to improve mitochondrial energy metabolism.

## 4.1. Introduction

Src family kinases (SFKs) are a major group of tyrosine (Tyr) kinases that are essential for the regulation of a large number of intracellular signaling pathways, including cell growth and proliferation, differentiation, motility, adhesion, cell death, and survival, in a variety of cell types (Parsons & Parsons, 2004; Thomas & Brugge, 1997). Aberrant expression of SFK members, such as c-Src, have been shown to induce malignant properties in cells, thereby establishing them as proto-oncogenes (Blume-Jensen & Hunter, 2001; Irby & Yeatman, 2002; M. P. Kim et al., 2009). In fact, the increased expression and/or activity of c-Src kinase has been observed in colorectal, ovarian, lung, breast, and liver cancer (Djeungoue-Petga et al., 2019; Irby & Yeatman, 2000; Rosen et al., 1986; Yeatman, 2004), including human hepatocellular carcinoma (HCC) which is the most common type of liver cancer and one of the most malignant and metastatic cancers worldwide (Masaki et al., 1998; R. Zhao et al., 2015).

c-Src is one of the ubiquitously expressed SFK members with a high sequence homology to Yes and Fyn and, consequently, has overlapping functions in cellular pathways (E. C. Koc et al., 2016; Thomas et al., 1995). c-Src, as well as several other SFKs, Fyn, Fgr, Lyn, and Lck, are localized to the mitochondria and regulate mitochondrial pathways (Hebert-Chatelain, 2013; Salvi et al., 2002; Tibaldi et al., 2008; Vahedi et al., 2015), such as pyruvate decarboxylation (Y. Jin et al., 2016), the tricarboxylic acid (TCA) cycle (Lewandrowski et al., 2008; Salvi et al., 2007), apoptosis (Lopez et al., 2012), and oxidative phosphorylation (OXPHOS) (Acin-Perez et al., 2014; Augereau et al., 2005; Demory et al., 2009; Hebert-Chatelain et al., 2012; Lewandrowski et al., 2008; Miyazaki et al., 2003; Ogura et al., 2012; Salvi et al., 2007). OXPHOS is supported by four electron transfer chain complexes, complex I-IV and ATP synthase, complex V. The majority of these complexes have been shown to be Tyr

phosphorylated by SFKs; however, c-Src was identified as the kinase responsible for the phosphorylation of two nuclear-encoded subunits, NDUFB10 and NDUFV2, of complex I, SDHA of complex II, and the mitochondrial-encoded subunit, COII, of complex IV (Demory et al., 2009; Hebert-Chatelain et al., 2012; Miyazaki et al., 2003; Ogura et al., 2012). The phosphorylation of COII by c-Src kinase and the epidermal growth factor receptor (EGFR) were shown to regulate the activity of complex IV as well as cellular ATP synthesis in several normal and cancer cell lines (Demory et al., 2009). In addition to the alteration of OXPHOS activity by SFK phosphorylation of its complexes, our laboratory recently demonstrated the implication of Fyn with mitochondrial translation which is responsible for the synthesis of the 13 core subunits of OXPHOS complexes I, III, IV, and V (E. C. Koc et al., 2016). Although reduced mitochondrial function and c-Src activity have been implicated in promoting the Warburg effect in various cancer types (Y. Jin et al., 2016; Wallace, 2005, 2012), the role of c-Src in mitochondrial energy metabolism in liver cancer still remains to be established.

In this study, we investigated the role of c-Src on mitochondrial energy metabolism by studying the steady-state expression of OXPHOS complex subunits. We observed increased expression of c-Src in metastatic liver tumors and the metastatic liver cancer cell line, Hep3B, which was correlated with significant reductions in the expression and activity of OXPHOS complex I and IV subunits. Additionally, we observed the same association between high c-Src expression and impaired OXPHOS complex expression and activity in mouse embryonic fibroblast (MEF) cell lines. Interestingly, the inhibition of c-Src with the SFK inhibitor PP2 stimulated the expression of complex I and IV subunits, improved their enzymatic activities, and significantly reduced cell proliferation in both cancer and normal cell lines. The suppression of c-Src using c-Src siRNA was also shown to increase mitochondrial OXPHOS expression. Our

studies strongly indicate that c-Src impairs the expression and function of OXPHOS complexes, which can be one of the underlying factors for the development and progression of liver cancer. Furthermore, our findings provide evidence that the inhibition of SFKs with PP2 can be used as possible therapeutic strategies to inhibit cell proliferation and improve mitochondrial function and energy metabolism in liver cancers.

## 4.2. Materials and Methods

### 4.2.1. Liver Tumor Samples

Liver tissue biopsies, five cancerous and four non-cancerous, were derived from patients treated at the Pennsylvania State University Health Milton S. Hershey Medical Center. Protein lysates used in Western blot analyses were obtained by the resuspension and sonication of tissues in RIPA buffer containing 1% SDS. Lysates were then separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and probed with various antibodies, as described below.

### 4.2.2. Cell Culture

Human liver cancer Hep3B and HepG2 cell lines and mouse embryonic fibroblast (MEF) cells with endogenous expression of c-Src, Yes, and Fyn (WT), MEF cells with functional null mutations in both alleles of c-Src, Yes, and Fyn kinases (SYF), MEF cells with an endogenous expression of c-Src kinase and null mutations in Yes and Fyn ($Src^{++}$), and SYF cells with a stable over expression of c-Src kinase (Src) were purchased from the American Type Culture Collection (ATCC). Monolayer cultures of each cell line were maintained in Dulbecco's modified Eagles Medium (DMEM) (Hyclone, Thermo-Scientific, Waltham, MA) with 4.5 g/L glucose and adjusted to contain 10% fetal bovine serum (FBS) (Rocky Mountain Biologicals, Missoula, MT), 4 mM glutamine, 1 mM pyruvate, and 1% penicillin/streptomycin (P/S) (Corning Cellgro, Manassas, VA). The cells were grown in a humidified incubator at 37 °C with

5% $CO_2$. Collected cells were treated with sodium orthovanadate to preserve Tyr phosphorylation (Hebert Chatelain et al., 2011; Nishikawa et al., 2009).

Transfections of small interfering ribonucleic acids (siRNAs) were performed using Lipofectamine transfection reagent according to the manufacturer's protocols (Thermo Fisher Scientific). Partial knock down of human and mouse c-Src were performed using control and c-Src siRNAs obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). The Hep3B, HepG2, WT, SYF, $Src^{++}$, and Src cell lines were also treated with the Src family kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine) (EMD Chemicals, Gibstown, NJ) (Hanke et al., 1996), dissolved in dimethyl sulfoxide (DMSO), at concentrations ranging from 0 to 10 μM. Briefly, cells were seeded at $25x10^4$ cells/mL in DMEM containing 0.2% FBS and incubated for 24 h. The media was then replaced with fresh DMEM containing 10% FBS and cells were treated with PP2 for 24 to 72 h. To measure cell proliferation, cells were grown as described above and counted using the Trypan blue exclusion assay. For reactive oxygen species (ROS) generation assays, cells were grown as described above and the amount of ROS produced by the Hep3B and HepG2 cells was measured using Amplex Red assays (Thermo Fisher Scientific) according to the manufacturer's protocol. The results for cell proliferation and ROS generation assays are represented as the mean ± SD of at least three experiments for each group and presented as a percentage of the control cells (HepG2 cells = 100%; control cells for treatments = 100%).

### 4.2.3. Western Blot Analyses

Whole cell lysates obtained from liver biopsies and cell lines were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, and protease and phosphatase inhibitor cocktails. Protein

concentrations were determined using the bicinchoninic protein assay (BCA) (Pierce, Rockford, USA). The protein lysates (10 – 40 μg) were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (Amersham, GE Healthcare, Pittsburg, PA), stained with Ponceau S to ensure equal protein loading, blocked in a Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST), 5% (w/v) dry skim milk, and 1% BSA, and incubated with the corresponding primary antibodies overnight. Human and rodent OXPHOS complex cocktails and SDHA antibodies were purchased from Sigma Aldrigh (St. Louis, MO). Citrate synthase (CS), acyl-coenzyme A oxidase 1 (ACOX1), and c-Src kinase antibodies were purchased from Santa Cruz (Dallas, TX). Phosphotyrosine (PY-100), phospho-Src family (pSFK), LDHA, and PDH antibodies were purchased from Cell Signaling Technologies (Danvers, MA). The pSFK antibody detects the phosphorylation of c-Src and other family members Fyn, Lyn, Lck, Yes, and Hck at the kinase activation site Tyr416 (pTyr416) or the equivalent residue. The loading control antibody GAPDH was purchased from Fitzgerald (Acton, MA). Mitochondrial EF-Tu and TFAM antibodies were kind gifts from Dr. Linda Spremulli and Dr. Craig Cameron, respectively. The protein immunoreactivity was detected using the ECL Western blotting detection kit from Amersham (GE Healthcare, UK) and the membranes were developed per the manufacturer's protocol. The membranes were exposed to autoradiography films and band intensities were quantified using UN-Scan-It (Silk Scientific, Inc, Orem, UT) and ImageJ (Schneider, C. A., Rasband, & Eliceiri, 2012). The quantified values were normalized to the protein loading determined by Ponceau S staining and GAPDH antibody probing of the membranes. The expression of each protein was quantified from individual tissue and cell lysates from at least three independent experiments. The results represent the mean ± SD for each

quantified sample and are expressed as a percentage of the control cells (non-cancerous liver tissues = 100%; HepG2 cells = 100%; SYF cells = 100%; control cells for treatments = 100%).

#### 4.2.4. Mitochondrial OXPHOS Complex Enzymatic Activity Assays

Cell pellets were dissolved in a buffer containing 10 mM Tris-HCl (pH 7.0), 250 mM sucrose, and phosphatase and protease inhibitor cocktails. Briefly, for the complex I and III enzymatic activity assay, cell lysates were further diluted in a 50 mM phosphate buffer (pH 7.4) containing 1 mM potassium cyanide, 1 mg/mL BSA, and 100 µM cytochrome *c*. Protein concentrations were determined using the BCA protein assay and protein amounts were normalized by Ponceau S staining and GAPDH antibody probing of the membranes. The reactions were started by adding 200 µM NADH and the combined activity of complex I and III was determined spectrometrically by measuring the reduction of cytochrome c at 550 nm. The complex IV enzymatic activity protein lysates were diluted in a 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 100 µM reduced cytochrome c. The activity of complex IV was also measured spectrometrically by monitoring cytochrome c oxidation at 550 nm, as previously described (Birch-Machin & Turnbull, 2001). The rate of cytochrome c reduction and oxidation were calculated by dividing the change in absorbance at 550 nm between two linear points by the difference in time using the formula: Rate = (Abs 1 – Abs 2) / (Time 1 – Time 2). Each sample was run in triplicate for each experiment. The data are expressed as the mean ± SD of at least three experiments and are presented as a percentage of the control (HepG2 cells = 100%; SYF cells = 100%; control for treated cells = 100%).

#### 4.2.5. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.07. Statistically significant differences between non-cancerous/cancerous liver biopsies, HepG2/Hep3B cell lines, MEF

(WT/SYF//Src$^{++}$/Src) cell lines, and control/treated cells were detected using unpaired Student's *t*-tests (2-tailed), *P < 0.05. The non-cancerous tissues, HepG2, SYF, and control cells for treatments were set to 100% during statistical analyses. All data are expressed as mean ± SD, unless otherwise described.

## 4.3. Results and Discussion

### 4.3.1. c-Src is Overexpressed in Metastatic Liver Cancers

Aberrant expression and activity of c-Src was reported in patients with HCC and was positively correlated with tumor stage and metastasis. Thus, c-Src is believed to play an important role in the malignant transformation of hepatocytes (Masaki et al., 1998; R. Zhao et al., 2015). Although c-Src has been located in the mitochondria and established as one of the major regulators of mitochondrial energy metabolism and OXPHOS in various cell lines (Salvi et al., 2002; Tibaldi et al., 2008) and has been identified as a promoter of the aggressiveness of breast cancer cells by reduced mitochondrial activity (Djeungoue-Petga et al., 2019), the effects of c-Src on the expression of OXPHOS complexes in liver cancer have yet to be determined. Therefore, a better understanding of c-Src signaling and mitochondrial energy metabolism in liver cancer is essential to improve disease prognosis and treatments. To investigate c-Src kinase in liver cancer, we examined and compared the expression of c-Src in two groups of liver biopsies, cancerous and non-cancerous (patient tissue characteristics described in Table 4), by Western blot analyses. The expression of c-Src was detected in all liver tissues with a significantly higher expression of approximately 61% in the cancerous tissues compared to the non-cancerous tissues (Fig. 21). We also assessed the activation of c-Src by observing its phosphorylation at Tyr416, which is located in the activation loop of the kinase domain and is responsible for the enzymatic activity of c-Src when phosphorylated (T. Hunter, 1987; Thomas

& Brugge, 1997). The phospho-Src family (pSFK) antibody detects the endogenous c-Src phosphorylation at Tyr416, in addition to the phosphorylation of other SFK members, Lyn, Fyn, Lck, Yes, and Hck, at equivalent activation sites. An increase in SFK phosphorylation was also detected in liver cancer biopsies with an increased phosphorylation in cancerous tissues, especially those correlated with a higher c-Src expression (Fig. 21). The SFK phosphorylation was increased in the non-cancerous tissue from patient 7; however, this increase in pSFK may be due to the detection of another active SFK member. Interestingly, the tissues with the highest expression of c-Src and pSFK were metastatic adenocarcinomas (Table 4) from patients 2, 3, and 5 (Fig. 21), implying that increased c-Src expression and activity may contribute to the metastatic state of the tumors.

Subsequently, the steady-state expression of OXPHOS complex subunits was also examined in the two tissue groups using an antibody cocktail that detects the nuclear-encoded subunits of complex I (NDUFB8), II (SDHB), III (UQCRC2), and V (ATP5A) and the mitochondrial-encoded subunit of complex IV (COII) (Fig. 21), along with an antibody for complex II (SDHA). The lysates were also probed with GAPDH antibody as a protein loading control. Compared to the non-cancerous biopsies, the expression of complex III and V subunits (UQCRC2 and ATP5A, respectively) were reduced in the cancerous tissues, while the subunits of complexes I (NDUFB8) and II (SDHA and SDHB) were significantly decreased by roughly 55%, 45%, and 25%, respectively, as shown in Fig. 21. The expression of the mitochondrial-encoded complex IV subunit (COII) was also substantially decreased by approximately 35% in the cancerous liver tissues (Fig. 21). Surprisingly, the lowest expression of OXPHOS complexes was also identified in the metastatic tissues from patients 2, 3, and 5 (Fig. 21), potentially implicating impaired OXPHOS complex expression in cancer metastasis. The altered expression

of both nuclear- and mitochondrial-encoded OXPHOS subunits implies that there may be a possible defect in protein synthesis. Due to these findings, we investigated two factors essential to the synthesis of mitochondrial proteins. The expression of both mitochondrial transcription factor A (TFAM) and mitochondrial elongation factor Tu (EF-Tu) was comparable between the two tissue types, signifying that the synthesis of nuclear- and mitochondrial-encoded OXPHOS subunits may be regulated by c-Src kinase. These results strongly suggest that increased c-Src expression is correlated with reduced OXPHOS subunit expression and may play a role in liver cancer.

To further examine the impaired OXPHOS complex expression observed in the cancerous liver tissues above, we analyzed the expression of several crucial metabolic enzymes that control OXPHOS by regulating pathways such as the citric acid cycle (CAC), fatty acid beta oxidation, and pyruvate metabolism, including peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), citrate synthase (CS), pyruvate dehydrogenase (PDH), as well as mitochondrial markers heat shock protein 60 (HSP60) and voltage-dependent anion channel (VDAC)/porin. The expression of ACOX1, the first enzyme of the fatty acid beta oxidation pathway, is significantly decreased in the cancerous tissues from patients 2, 3, and 5, indicating that fatty acid beta oxidation is reduced in these patients which possibly implies lower energy production through oxidative phosphorylation, as observed by reduced expression of OXPHOS complexes (Fig. 22). Next, we also examined the expression of citrate synthase, one of the major checkpoints for the CAC. CS expression was comparable between the cancerous and non-cancerous liver tissues (Fig. 22) suggesting that the CAC activity is relatively equal between the two tissue types. Due to these findings, we further analyzed other metabolic pathways and enzymes that converge onto the CAC, such as PDH. The expression of PDH, which is a critical

enzyme involved in the regulation of pyruvate metabolism by catalyzing the conversion of pyruvate to acetyl-CoA and connecting glycolysis to the CAC and mitochondrial oxidative metabolism. The expression of PDH was higher in the cancerous tissues of patients 2, 3, and 5 (Fig. 22), implying that pyruvate metabolism is high in liver cancer tissues. Interestingly, c-Src expression, which has been shown to phosphorylate, inactivate PDH, and cause a restraint on the pyruvate flux into mitochondrial metabolism (Y. Jin et al., 2016), was increased in these cancerous liver tissues (Fig. 21). Therefore, although the expression of PDH was increased, the presence of high c-Src expression may inhibit the activity of PDH, causing these metastatic tissues to rely more on glycolysis than mitochondrial oxidative metabolism, the Warburg effect. Combined, our results suggest that c-Src may be contributing to the metabolic reprogramming of cancer tissues and inducing cancer metastasis by impairing mitochondrial oxidative phosphorylation.

To ensure the reduction in the expression of OXPHOS complexes was not a result of decreased mitochondrial presence, we assessed the expression of two mitochondrial markers, VDAC/porin and HSP60. The expression of VDAC/porin, found in the mitochondrial outer membrane, was comparable between cancerous and non-cancerous tissues, implying comparable amounts of mitochondria between each liver tissue analyzed (Fig. 22). The expression of HSP60, a mitochondrial chaperone protein that is responsible for the folding and assembly of newly imported proteins in the mitochondria, is reduced (Fig. 22). Additionally, minor differences were observed in the expression of mitochondrial matrix protein, CS, between the cancerous and non-cancerous liver tissues, demonstrating that mitochondrial content was almost equal levels in both tissue types. These findings suggest that the decrease in OXPHOS complex expression was largely a result of the expression and activation of c-Src kinase.

| Patient | Age | Tissue type | Metastasis | Disease state |
|---|---|---|---|---|
| 1 | 18 | T | + | Adenocarcinoma |
| 2 | 20 | T | + | Adenocarcinoma |
| 3 | 28 | T | + | Adenocarcinoma |
| 4 | 31 | T | - | Adenoma |
| 5 | 43 | T | + | Adenocarcinoma |
| 6 | 58 | N | - | Benign cyst |
| 7 | 36 | N | - | Hyperplasia |
| 8 | 69 | N | - | Bilary cystadenoma |
| 9 | 73 | N | - | Macrovesicular steatosis |

**Table 4. Characterization of liver tumor and non-cancerous tissues**.
T: primary tumor
N: non-cancerous tissue

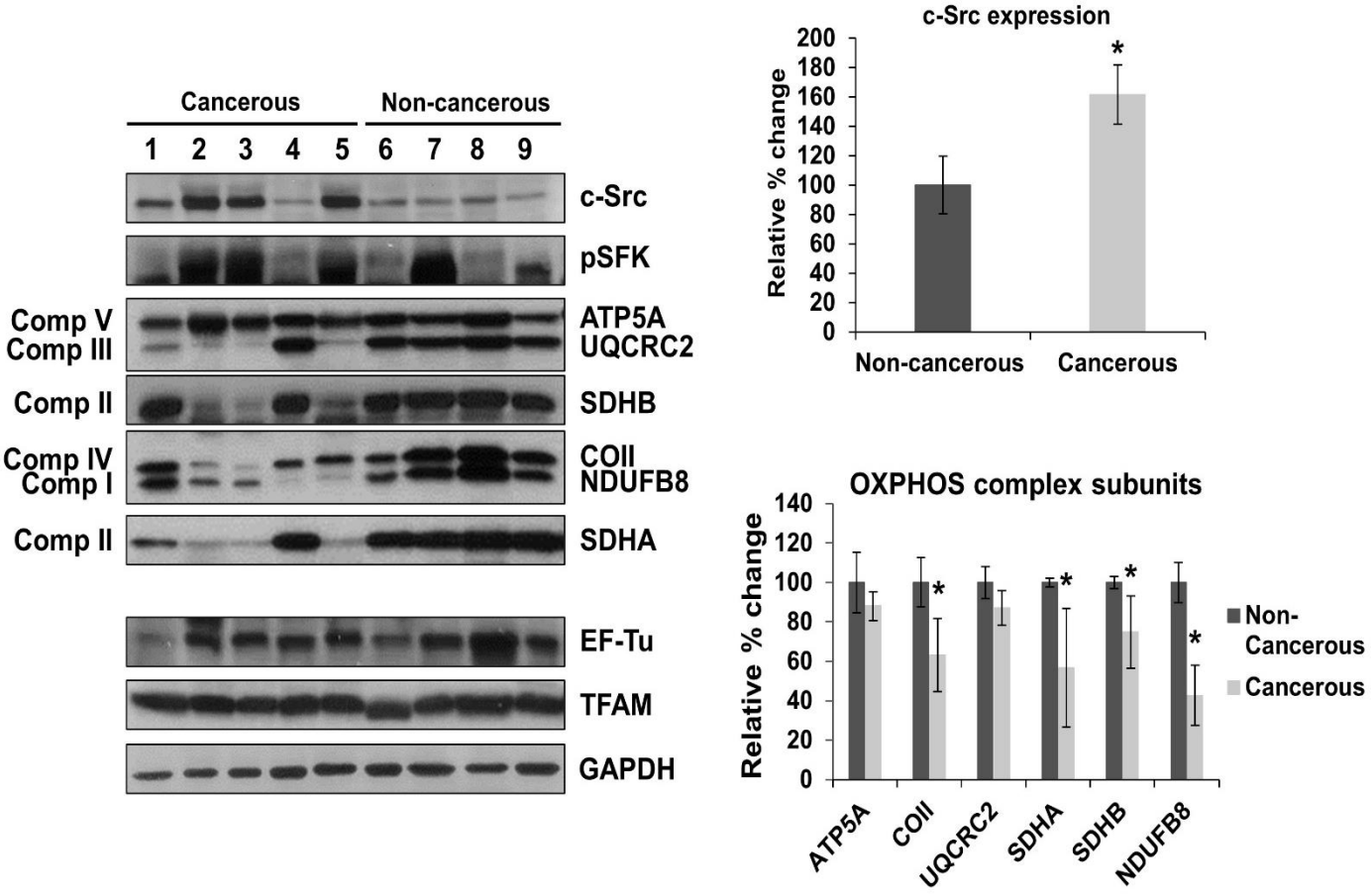


**Figure 21. c-Src expression is increased in metastatic liver cancer tissues.** The expression of OXPHOS subunits, including NDUFB8 (complex I), SDHA and SDHB (complex II), UQCRC2 (complex III), COII (complex IV), and ATP5A (complex V) were detected by Western blot analyses. The expression of c-Src kinase, mitochondrial transcription factor A (TFAM), and mitochondrial elongation factor Tu (EF-Tu) were also detected. Approximately 30 µg of whole cell lysates from each tissue was separated by 12% SDS-PAGE, and equal protein loading was determined by GAPDH antibody and Ponceau S staining of the membranes. The bar graphs represent the average quantitative analyses from densitometry of the expression of c-Src (top right panel) and the OXPHOS complex subunits (bottom right panel) of the two groups. Significant differences were identified in c-Src (P=0.0488), COII (P=0.0028), SDHA (P=0.0253), SDHB (P=0.0001), and NDUFB8 (P=0.0001). The data is represented as the mean ± SD of at least three experiments for each tissue group and are presented as a percentage of the non-cancerous tissues (non-cancerous tissues = 100%) (see Table 4 for details). Significant differences were observed between the non-cancerous and cancerous tissues via unpaired Student's *t*-tests (2-tailed), *P < 0.05.

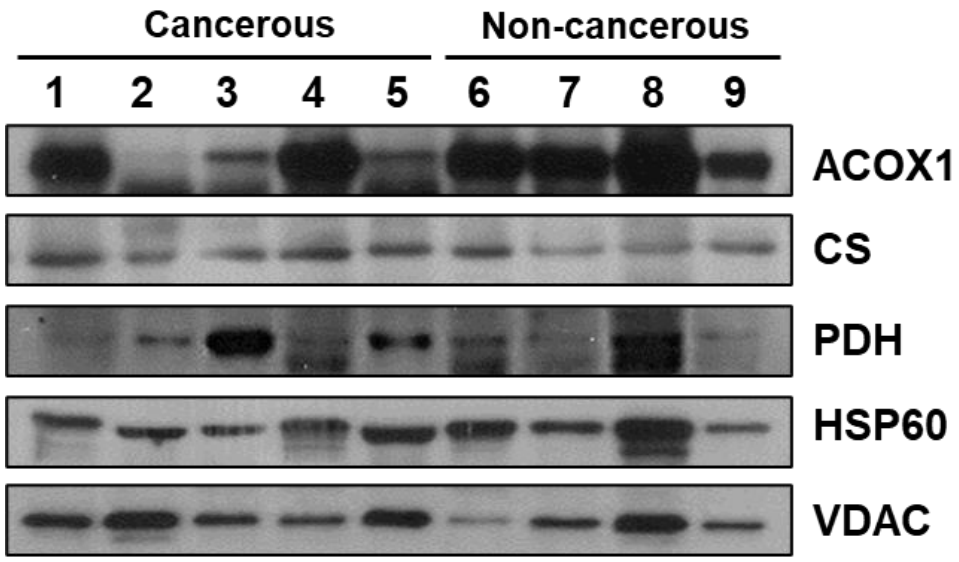


**Figure 22. Metabolic pathways are altered between cancerous and non-cancerous liver tissues**. The expression of peroxisomal acyl-coenzyme A oxidase (ACOX1), citrate synthase (CS), pyruvate dehydrogenase (PDH), heat shock protein 60 (HSP60), and voltage-dependent anion channel (VDAC)/porin were detected by Western blot analyses. See Fig. 21 legend for details.

### 4.3.2. c-Src is Expressed in Metastatic Liver Cancer Cell Line

The findings from the liver biopsies led us to further explore the relationship between c-Src and OXPHOS complex expression by studying their expressions in whole cell lysates from two of the most common liver cancer cell lines, Hep3B and HepG2, which are isolated from patients with primary HCC and hepatoblastoma (HB), respectively (Lopez-Terrada, Cheung, Finegold, & Knowles, 2009; Qiu et al., 2015; Slany et al., 2010). HepG2 cells express proteins that are characteristic of normal hepatocytes, while proteins expressed in Hep3B cells are involved in the induction of the epithelial mesenchymal transition (EMT) (Slany et al., 2010). Due to the increased expression of c-Src in the metastatic liver biopsies, we first assessed its expression in both cell lines. c-Src expression was clearly increased in Hep3B cells compared to almost no expression in HepG2 cells (Fig. 23A). Surprisingly, we found relatively similar SFK phosphorylation between Hep3B and HepG2 cell lines (Fig. 23A), indicating other SFK members are active in the two cell lines. Since a handful of SFKs have been located in the mitochondria (Salvi et al., 2002; Tibaldi et al., 2008), we analyzed the expression of some of these kinases. The expression of Lyn was comparable between the two cell lines (data not shown), while the expression of Lck and Fyn (data not shown) were increased in HepG2 cells, as previously described (E. C. Koc et al., 2016). Therefore, the expression of Lyn, Lck, Fyn, c-Src, and other SFKs contribute to the pSFK signal detected in Hep3B and HepG2 cells. Yet, our results clearly show c-Src expression is only found in the metastatic Hep3B cell line.

The mitochondrial localization of c-Src has been shown to increase (Arachiche et al., 2008; Ogura et al., 2012) or decrease (Demory et al., 2009; Y. Jin et al., 2016) the OXPHOS activity and expression in various cell lines. Due to our findings in the liver biopsies and the expression of c-Src in the Hep3B liver cancer cell line only, we investigated the relationship

between c-Src and changes in mitochondrial energy metabolism by observing the expression of OXPHOS subunits. Similar to what was found in the metastatic cancerous liver tissues from patients 2, 3, and 5 (Fig. 21), the subunits of complexes II, III, and V were decreased in Hep3B cells compared to HepG2 cells (Fig. 23A), with significant reductions in the expression of the nuclear-encoded subunit of complex I and the mitochondrial-encoded subunit of complex IV (NDUFB8 and COII, respectively) in Hep3B cells (Fig. 23A). Even with a lower expression of mitochondrial-encoded COII, the expression of EF-Tu and TFAM were comparable between the two cell lines (Fig. 23A). These results imply that c-Src may regulate the expression of both nuclear- and mitochondrial-encoded OXPHOS subunits in liver cancer tissues and cell lines.

Next, we determined the effects of the reduced expression of NDUFB8 and COII on mitochondrial oxidative phosphorylation and energy metabolism by performing complex I and III and complex IV enzymatic activity assays in both cell lines. As shown in Fig. 23B, a significant decrease of approximately 60% was observed in the complex I and III activity and a reduction of about 50% was found in the complex IV activity in Hep3B cells compared to HepG2 cells. The impaired mitochondrial enzymatic activities of complexes I and III and complex IV in Hep3B cells can be attributed to the reduction of the expression of nuclear-encoded NDUFB8 and mitochondrial-encoded COII, which are two core subunits of complexes I and IV, respectively. The reduced COII expression and activity are in agreement with previous studies that demonstrate reduced complex IV activity due to the phosphorylation of COII by c-Src and EGFR (Boerner et al., 2004; Demory et al., 2009). Since reactive oxygen species (ROS) are major byproducts of oxidative phosphorylation, we examined the ROS production between Hep3B and HepG2 cell lines. ROS generation was significantly reduced in Hep3B cells, which is possibly a result of the decreased OXPHOS expression and activity found in Hep3B cells.

Although Hep3B cells have significantly lower OXPHOS function, cell proliferation was increased compared to HepG2 cells.

To further study the decrease in OXPHOS in Hep3B cells, we analyzed the expression of several metabolic enzymes that control OXPHOS. The expression of ACOX1 was increased in Hep3B cells compared to HepG2 cells, demonstrating increased fatty acid oxidation in these cells (Fig. 23C). The expression of CS, PDH, and lactate dehydrogenase A (LDHA) were decreased in Hep3B cells implying that the reduction in oxidative phosphorylation may be a result of impaired CAC and pyruvate metabolism and further suggesting that Hep3B cells may rely more on anaerobic glycolysis for energy metabolism. In fact, both PDH and LDHA have known to be phosphorylated by c-Src and induce a more metastatic phenotype in various cancers (L. Jin et al., 2017; Y. Jin et al., 2016; J. Zhang et al., 2017), similar to our observation in Hep3B cells. Reductions in the expression of these metabolic enzymes could be caused by the diminished OXPHOS subunit expression and/or activity in the Hep3B cell line. Although the expression of mitochondrial marker, HSP60, is comparable between Hep3B and HepG2 cells indicating similar mitochondrial presence in the two cell lines, mitochondrial OXPHOS is regulated by c-Src kinase expression and activity. Along with an aberrant activity of c-Src, decreased expression of OXPHOS complexes I and IV and reduced mitochondrial function have been correlated with cancer metastasis and the induction of EMT (Gaude & Frezza, 2016; Porporato et al., 2014). The expression of several EMT factors analyzed were increased in Hep3B compared to HepG2 cell lines (data not shown), confirming the metastatic state of Hep3B cells, as previously described (Qiu et al., 2015; Slany et al., 2010). Furthermore, our findings suggest that increased c-Src expression may impair OXPHOS expression and activity which could contribute to the metastatic phenotype of the Hep3B cell line.

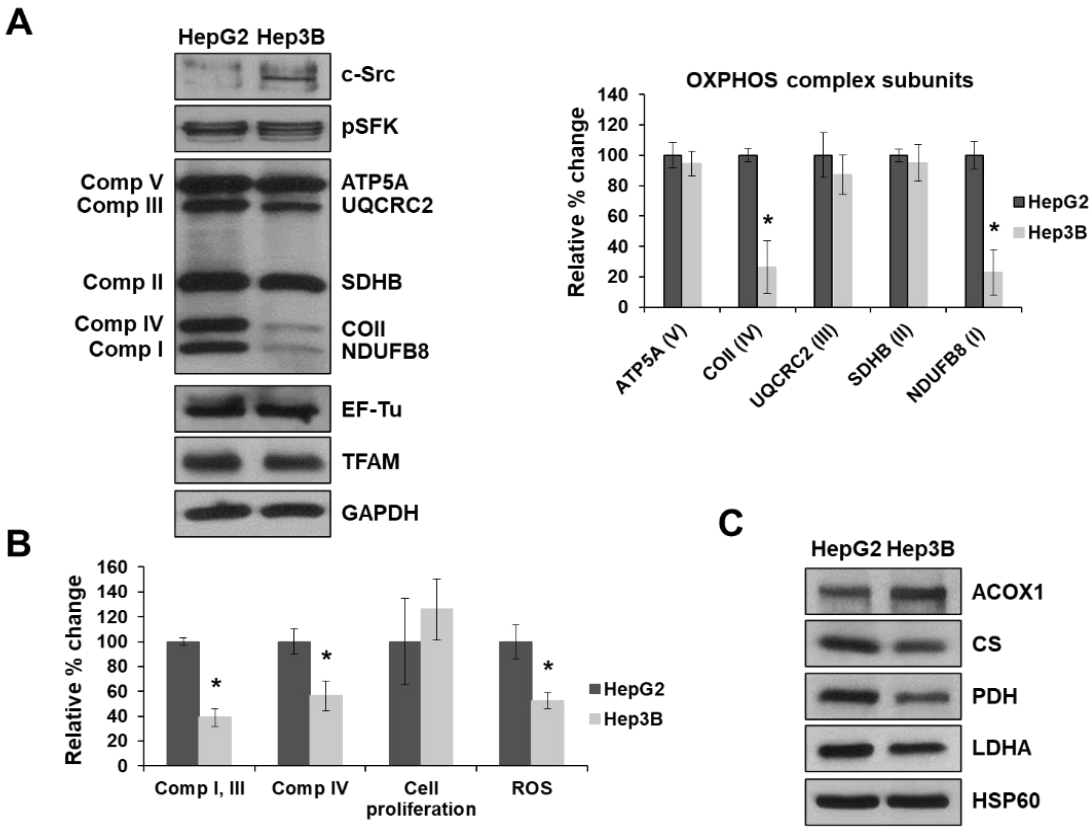


**Figure 23. c-Src is overexpressed in liver cancer cell lines**. **A)** The expression of OXPHOS complex subunits, c-Src kinase, and mitochondrial proteins TFAM and EF-Tu were detected by Western blot analyses in Hep3B and HepG2 cell lines. Additionally, the phosphorylation of SFK members at the activation site, Tyr416 (pSFK), was also observed. Equal protein concentration was determined by GAPDH antibody expression and Ponceau S staining of the membranes. Quantification of the average expression of OXPHOS complex subunits between the two cell lines is shown in the bar graph (right panel). The results represent the mean ± SD of at least three independent experiments. Significant difference was observed between HepG2 and Hep3B cells in OXPHOS subunits COII (P = 0.0104) and NDUFB8 (P = 0.0001). The data is presented as a percentage of HepG2 protein expression (HepG2 cells = 100%). **B)** HepG2 and Hep3 cells were grown for 48 h and proliferation was measured by the Trypan blue exclusion assay (cell proliferation). Combined complex I and III activity was determined by measuring the rate of cytochrome c reduction at 550 nm using equal amounts (10 µg) of whole cell lysates obtained from HepG2 and Hep3B cells. Complex IV enzymatic activity was determined by measuring the rate of cytochrome c oxidation spectrometrically at 550 nm using equal amounts of HepG2 and Hep3B whole cell lysates. ROS generation was assessed with the Amplex Red assay using equal concentrations of HepG2 and Hep3B cell lysates. Significant differences were observed between Hep3B and HepG2 cells in complex I and III activity (P = 0.0001), complex IV activity (P = 0.0139), cell proliferation (P = 0.013), and ROS generation (P = 0.0002). **C)** The expression of metabolic enzymes peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), citrate synthase (CS), pyruvate dehydrogenase (PDH), lactate dehydrogenase A (LDHA), and heat shock protein 60 (HSP60) were detected by Western blot analyses of HepG2 and Hep3B cell lines. See Fig. 21 legend for details. Values are presented as mean ± SD for at least three experiments and presented as a percentage of HepG2 (HepG2 = 100%). Significant differences were observed between Hep3B and HepG2 cells via unpaired student's *t*-test (2-tailed) with Welch's correction, *P < 0.05.

### 4.3.3. Inhibition of c-Src Stimulates the Expression of OXPHOS Subunits

Our data provide strong evidence that c-Src expression is associated with impaired OXPHOS complex expression and activity in liver cancer cells. Since aberrant expression of c-Src was shown in metastatic liver cancer tissues and cell lines (Figs. 21 and 23) and has been correlated with cancer metastasis, recurrence, adverse prognosis, and resistance to therapies (Yeatman, 2004; S. Zhang et al., 2011; R. Zhao et al., 2015), we investigated c-Src kinase as a potential target for liver cancer therapies by treating Hep3B and HepG2 cell lines with the selective SFK inhibitor PP2 at concentrations ranging from 0 – 10 μM for 72 h and investigated the steady-state expression of OXPHOS complex subunits. PP2 is a membrane-permeable inhibitor that blocks the ATP binding site of c-Src and prevents activation without altering its expression (Hanke et al., 1996; Hebert-Chatelain, 2013).

We first determined the effect of PP2 treatments on c-Src kinase in Hep3B cells and observed a substantial decrease of approximately 40% in phosphorylation at Tyr416 (pSFK) using the pSFK and the phospho-Tyr antibody, along with a reduction in the overall Tyr phosphorylation at PP2 concentrations ranging from 0 to 5 μM (Fig. 24A). Although SFK phosphorylation was lower, minor differences in the expression of c-Src were observed in the presence of PP2 (Fig. 24A). Interestingly, the suppression of c-Src kinase stimulated the expression of nuclear-encoded NDUFB8 and mitochondrial-encoded COII in a concentration-dependent manner, with a significant increase of approximately 50% in their expression at 5 μM PP2, as shown in Fig. 24A. However, minor changes were found in most of the nuclear-encoded OXPHOS complex subunits, SDHB, UQCRC2, and ATP5A in the presence of PP2 (Fig. 24A). Despite the higher expression of COII and NDUFB8, the expression of EF-Tu and TFAM were comparable between the treated and control cells (Fig. 24A), demonstrating that the increased

expression of OXPHOS subunits may be a result of c-Src inhibition and decreased Tyr phosphorylation. To determine if the increases in the expression of NDUFB8 and COII altered the function of complexes I and IV, respectively, we performed complex I and III and complex IV enzymatic activity assays. Significant increases were measured in the complex I and III activity of Hep3B cells treated with 2.5 and 5 μM PP2 (Fig. 24B). Similar increases were found in the complex IV activity of PP2 treated Hep3B cells (Fig. 24B). Additionally, the proliferation of Hep3B cells was decreased by approximately 65% and 80% at 2.5 and 5 μM PP2, respectively (Fig. 24B). The results of these studies demonstrated that the inhibition of c-Src kinase by PP2 stimulated OXPHOS complex expression which may be therapeutically beneficial to increase mitochondrial energy metabolism and possibly contribute to the decrease in cell proliferation.

In addition to Hep3B cells, we treated the HepG2 cell line with PP2 at concentrations ranging from 0 to 10 μM for 72 h. Minor differences were found in the expression of OXPHOS complex subunits in HepG2 treated cells compared to the control cells (Fig. 25). Yet, we showed a clear reduction in the proliferation of HepG2 cells, which was associated with reduced SFK phosphorylation at increasing concentrations of PP2 (data not shown). Although PP2 treatments reduced cell proliferation in both Hep3B and HepG2 cell lines by suppressing phosphorylation at Tyr416, the increase in OXPHOS complex subunits COII and NDUFB8 were only observed in Hep3B cells. These findings strongly imply that c-Src impairs OXPHOS expression, which can be alleviated when c-Src activity is suppressed.

As shown in Fig. 24C, siRNA mediated knock down of c-Src kinase reduced c-Src expression and activity relative to the cells transfected with control siRNA in Hep3B cells. The expression of NDUFB8 and COII of complexes I and IV, respectively, were slightly increased by approximately 40% in cells transfected with c-Src siRNA (Fig. 24C). The expression of SDHB

and UQCRC2, of complexes II and III, respectively, were also increased; however, the expression of ATP5A of complex V remained unchanged (Fig. 24C). Minor differences were observed in the expression of TFAM and EF-Tu (data not shown), implying that the increase found in the expression of OXPHOS complexes may be a result of the suppression of c-Src. The increase in the expression of NDUFB8 and COII in Hep3B cells transfected with c-Src siRNA is similar to what was observed in PP2 treated Hep3B cells (Fig. 24A). These findings provide further evidence that c-Src inhibition stimulates OXPHOS expression and increases mitochondrial energy metabolism. Therefore, c-Src should be closely evaluated as a therapeutic agent for cancers with increased c-Src expression and activity.

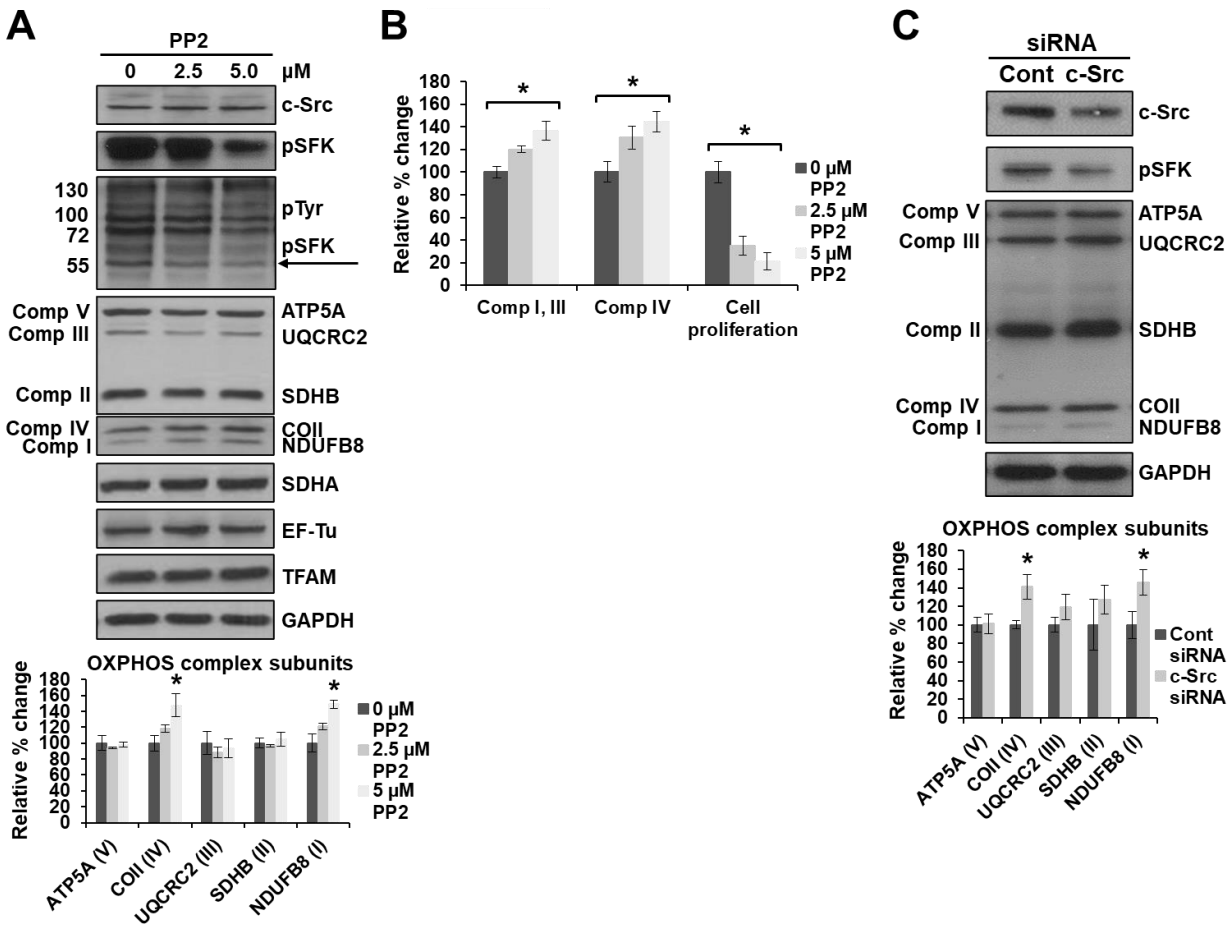


**Figure 24. Inhibition of c-Src increases OXPHOS expression in the Hep3B cell line**. **A)** Hep3B cells were treated with 0 – 5 µM Src family kinase (SFK) inhibitor PP2 for 72 h before collection. Changes in the expression of OXPHOS complex subunits, c-Src, pSFK, Tyr phosphorylation (pTyr), EF-Tu, and TFAM were measured by Western blot analyses with respect to GAPDH antibody probing and Ponceau S staining of the membranes as equal protein loading controls. The phosphorylation of SFK members (pSFK) was detected by the pTyr antibody, indicated by an arrow, as well as the pSFK antibody that detects phosphorylation at Tyr416. The quantitative analyses of the expression of OXPHOS subunits are represented as mean ± SD of at least three experiments for each group (bar graph, right panel). Significant differences were observed in the expression of COII ($P = 0.0091$) and NDUFB8 ($P = 0.024$) at 5 µM PP2. **B)** Cell proliferation of PP2 treated Hep3B cells were measured with the Trypan blue exclusion assays after 72 h treatment. Statistical difference was shown between the control and 2.5 µM ($P = 0.001$) and 5 µM ($P = 0.0001$) PP2. Complex I and III and complex IV activities were measured by determining the rates of cytochrome c reduction and oxidation, respectively, at 550 nm. Significant differences were observed in the complex I and III activity at 2.5 µM ($P = 0.0122$) and 5 µM ($P = 0.0006$) PP2 and complex IV activity at 2.5 µM ($P = 0.0002$) and 5 µM ($P = 0.0001$) PP2. **C)** Western blot analyses were used to identify changes in Hep3B cells transfected with with c-Src siRNA (c-Src) relative to control siRNA (cont). The quantitative analyses of OXPHOS expression are represented as the mean ± SD of at least three experiments for each group (bar graph, right panel). Significant differences were observed in the expression of COII ($P = 0.0397$) and NDUFB8 ($P = 0.0022$) between the control and c-Src siRNA transfected Hep3B cells. Data represents the mean ± SD of at least three independent experiments. The results are presented as a percentage of the control cells (cont = 100%). Statistical difference was observed between the control and treatment groups via unpaired Student's t-test (2-tailed), $*P < 0.05$.

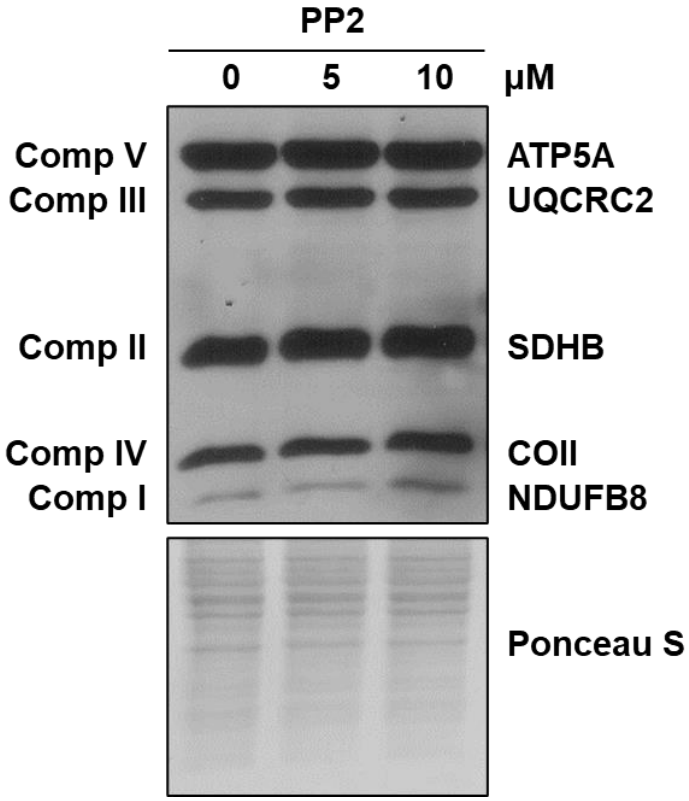


**Figure 25. PP2 treatments of HepG2 cells reduce cell proliferation without altering OXPHOS expression**. HepG2 cells were treated with 0 – 10 μM PP2 for 72 h before collection. Expression of OXPHOS complex subunits, including NDUF8 (complex I), SDHA and SDHB (complex II), UQCRC2 (complex III), COII (complex IV), and ATP5A (complex V) were detected by Western blot analyses. Approximately 30 μg of protein lysates from HepG2 cells treated with PP2 were separated by 12% SDS-PAGE and equal protein loading was assessed by Ponceau S staining and GAPDH antibody probing of the membranes.

### 4.3.4. c-Src Expression Impairs the Expression of OXPHOS Complexes

Above, we clearly showed increased c-Src expression was associated with impaired OXPHOS expression and activity, especially complexes I and IV, in metastatic liver cancer tissues and cell lines which was alleviated when c-Src activity was suppressed. Our findings imply that c-Src kinase regulates mitochondrial oxidative phosphorylation by altering the expression and activity of its complexes. To further examine the functional significance of c-Src on mitochondrial energy metabolism, we investigated changes in the expression of OXPHOS complexes in more homogenous cellular system. For this purpose, we used the mouse embryonic fibroblast (MEF) cell line (WT cells), MEF cells with functional null mutations in both alleles of c-Src, Yes, and Fyn (SYF cells), MEF cells with an endogenous expression of wild-type c-Src and null mutations in Yes and Fyn ($Src^{++}$ cells), and SYF cells with a stable over expression of wild-type c-Src kinase (Src cells). Detection of c-Src expression by Western blot analyses in all four cell lines revealed varying expression of c-Src in WT, $Src^{++}$ and Src cells (Fig. 26A) compared to its complete knock down in SYF cells. The pSFK level was significantly higher in $Src^{++}$ and Src cells compared to SYF cells with the highest phosphorylation in WT cells (Fig. 26A). The phosphorylation of Tyr416 in WT cells could be a result of the activation of c-Src as well as Yes, Fyn, and other SFKs expressed in WT cells. The levels of pSFK detection in WT, $Src^{++}$, and Src cell lines can also be correlated to the level of Tyr phosphorylation in these cell lines (Fig. 27) confirming both c-Src expression and activity in $Src^{++}$, Src, and WT cells compared to SYF cells.

We assessed the steady-state expression of OXPHOS subunits in the four cell lines using an OXPHOS antibody cocktail that detects subunits from nuclear-encoded complex I (NDUFB8), II (SDHB), III (UQCRC2), V (ATP5A), and the mitochondrial-encoded complex IV

(COI) (Fig. 26A). Significant reductions were observed in the expression of the nuclear-encoded complex I (NDUFB8) and mitochondrial-encoded complex IV (COI) subunits with increasing c-Src expression in WT, $Src^{++}$, and Src cells compared to the SYF cells (Fig. 26A). On the contrary, the expression of complex II, III, and V subunits were comparable among all the cell lines (Fig. 26A). These results strongly suggest that c-Src expression impairs the expression of complex I and IV subunits in mouse embryonic fibroblast cells.

To determine the effect of reduced NDUFB8 and COI expressions, we performed mitochondrial complex I and III and complex IV enzymatic activity assays in WT, SYF, $Src^{++}$, and Src cells. The activity of complex I and III was significantly decreased with an increasing expression of c-Src found in WT, $Src^{++}$, and Src cells compared to SYF cells (Fig. 26B). Similarly, the complex IV activity was also decreased in WT, $Src^{++}$, and Src cells compared to the SYF cell line (Fig. 26B). Therefore, the impaired mitochondrial function and diminished complex I and III and complex IV activities can be attributed to the reduced expression of the core subunits, NDUFB8 and COI, and the associated increase in the expression of c-Src in these cells.

c-Src kinase has also been shown to control cell proliferation, migration, and adhesion; consequently, we examined the cell proliferation of fibroblast cells with and without c-Src expression. In cells expressing c-Src, cell proliferation was significantly higher compared to SYF cells (Fig. 26B). Increased cell proliferation and c-Src activity are correlated with high glycolytic metabolism by inhibiting PDH activity and OXPHOS, as previously described (Y. Jin et al., 2016). Therefore, the increased c-Src expression could also cause inhibition of PDH activity, resulting in diminished OXPHOS as observed in MEF cell lines (Fig. 26). These findings are in agreement with our Hep3B cell line data shown above and further imply that c-Src alters the

enzymatic activity of OXPHOS complexes, possibly by changing the expression of their subunits. Together, our results obtained with liver biopsies and cell lines suggest that c-Src expression regulates mitochondrial function in both normal and cancerous cells.

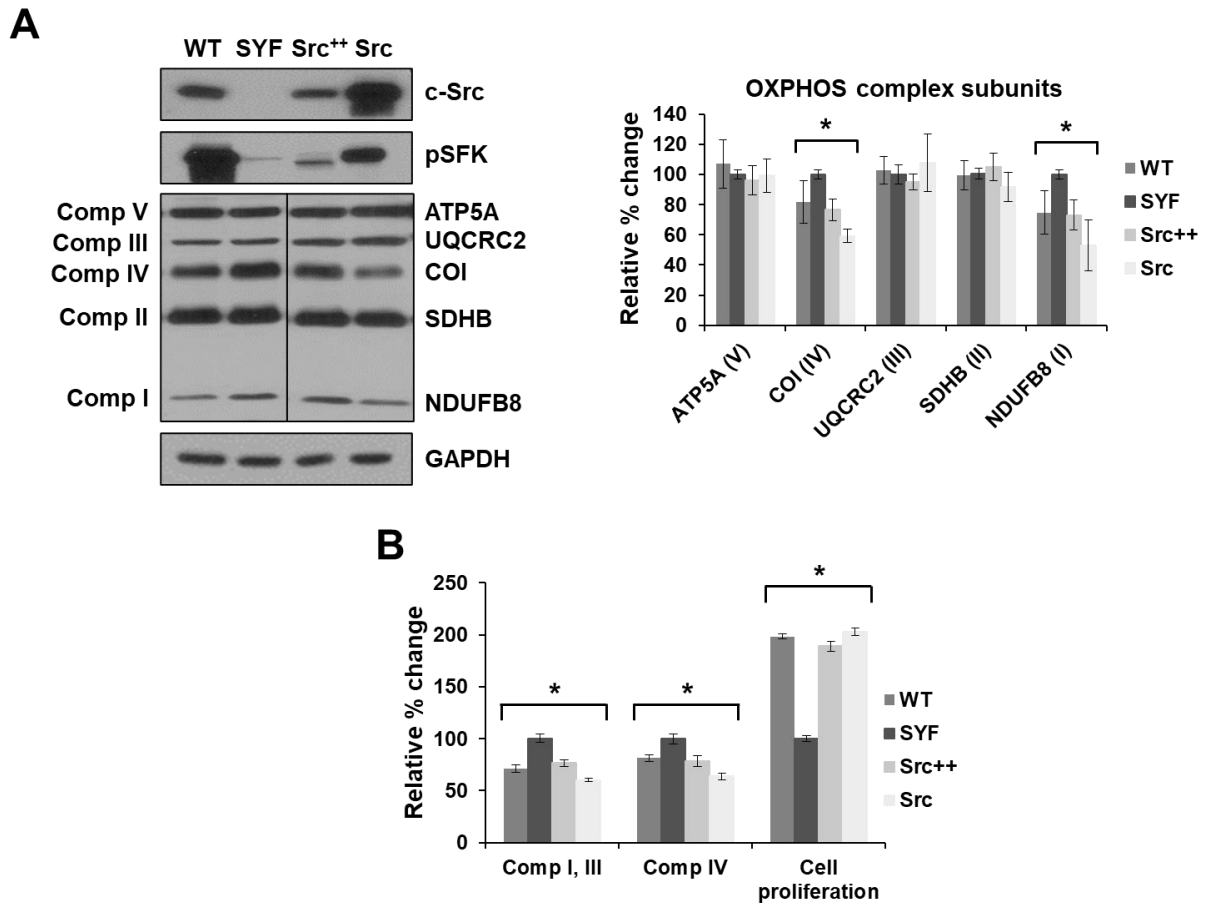


**Figure 26. c-Src expression reduces OXPHOS expression in fibroblast cells**. **A)** Mouse embryonic fibroblast (MEF) cells with functional null mutations in both alleles of three SFK members, c-Src, Yes, and Fyn (SYF), MEF cells with the endogenous expression of c-Src and null mutations in Yes and Fyn (Src++), SYF cells with the overexpression of c-Src (Src), and wild-type embryonic fibroblast cells (WT) were cultured. Expression of OXPHOS complex subunits, including NDUFB8 (complex I), SDHB (complex II), UQCRC2 (complex III), COI (complex IV), and ATP5A (complex V) were detected by Western blot analyses of WT, SYF, Src++, and Src cell lines. The expression of c-Src and pSFK were also detected with respect to GAPDH and Ponceau S staining as a control for equal protein loading. Quantification of the average expression of OXPHOS complex subunits between SYF and WT, Src++, and Src cells is shown in the bar graph (right panel). Significant differences were observed in the expression of COI between SYF and WT ($P = 0.0426$), Src++ ($P = 0.0017$), and Src ($P = 0.0001$) and in the expression of NDUFB8 between SYF and WT ($P = 0.0163$), Src++ ($P = 0.0033$), and Src ($P = 0.0016$). **B)** WT, SYF, Src++, and Src cells were grown for 24 h and proliferation was measured by the Trypan blue exclusion assay (cell proliferation). Significant differences were observed in the cell proliferation between SYF and WT ($P = 0.0007$), Src++ ($P = 0.0018$), and Src ($P = 0.0014$). The complex I and III and complex IV enzymatic activities were determined by measuring the reduction and oxidation rate, respectively, of cytochrome c using equal amounts of whole cell lysates obtained from WT, SYF, Src++, and Src cells. Significant differences were observed in the complex I and III activity between SYF and WT ($P = 0.0071$), Src++ ($P = 0.0001$), and Src ($P = 0.0002$) and complex IV activity ($P = 0.0001$). Results are expressed as mean ± SD for at least three experiments and are presented as a percentage of SYF cells (SYF = 100%). Significant differences were observed between SYF, WT, Src++, and Src cells via unpaired student's t-tests (2-tailed) to measure statistical significance, *$P < 0.05$. See Fig. 23 legend for details.

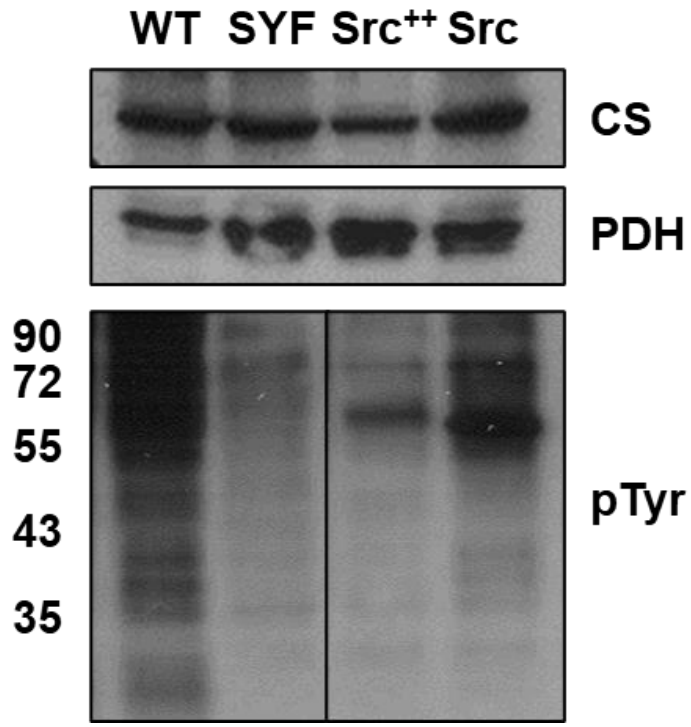


**Figure 27. Increased tyrosine phosphorylation is correlated with increased c-Src**. The overall tyrosine phosphorylation and the expression of citrate synthase (CS) and pyruvate dehydrogenase (PDH) were detected by Western blot analyses in WT, SYF, $Src^{++}$, and Src cells. See Fig. 26 legend for details.

### 4.3.5. c-Src Inhibition with PP2 Stimulates the Expression of OXPHOS Subunits

Our data provide strong evidence that c-Src expression is associated with impaired OXPHOS complex expression and activity in normal and cancerous cells. To investigate if the inhibition of c-Src kinase activity could improve OXPHOS subunit expression and function, we first treated MEF cell lines expressing the highest c-Src kinase, Src cells, with PP2 for 24 to 48 h. As expected, PP2 treatments decreased overall Tyr phosphorylation and the phosphorylation of c-Src at Tyr416 without influencing the c-Src expression (Fig. 28A). Interestingly, the steady-state expression of nuclear-encoded NDUFB8 and mitochondrial-encoded COI subunits of complexes I and IV, respectively, were significantly increased with PP2 treatments compared to the control cells, while minor changes were observed in the expression of the remainder of the OXPHOS subunits detected by the rodent antibody cocktail (Fig. 28A). Despite the higher expression in COI and NDUFB8, the expression of EF-Tu and TFAM (data not shown) were comparable, suggesting that the increase in the expression of OXPHOS subunits may be a result of the inhibition of c-Src kinase activity and the associated changes in Tyr phosphorylation.

Subsequently, significant increases of roughly 40% and 50% were observed in the enzymatic activities of complexes I and III and complex IV in PP2 treated Src cells, respectively, which may be a result of the stimulated expression of the NDUFB8 and COI subunits (Fig. 28B). In fact, the OXPHOS subunit expression and activity found in PP2 treated Src cells was comparable to the subunit expression of the SYF cell line (Figs. 26 and 28). The morphology of PP2 treated Src cells became similar to SYF cells (data not shown). Clearly, cell proliferation was also reduced by approximately 50% in PP2 treated Src cells compared to the control cells (Fig. 28B), further demonstrating the role of c-Src on cell proliferation and morphology.

In addition to the treatment of Src cells with PP2, we also treated the WT, $Src^{++}$, and SYF cell lines with PP2. Similar to what was observed in Src cells, we detected an increase in the expression of NDUFB8 and COI of complexes I and IV, respectively, in PP2 treated $Src^{++}$ cells (Fig. 29) and WT cells (data not shown). The increases in the expression of NDUFB8 and COI were correlated with a decrease in c-Src activity, determined by the changes in the pSFKs at Tyr416 and total Tyr phosphorylation. Additionally, increasing concentrations of PP2 were also correlated with significant decreases in cell proliferation in $Src^{++}$ (Fig. 29) and WT (data not shown) cell lines. To ensure the increases in the expression of OXPHOS complexes I and IV were a result of reduced c-Src activity, we treated SYF cell lines with PP2 for 48 h and observed the effects on OXPHOS complexes. Although the cell proliferation was diminished in SYF cells treated with PP2, the OXPHOS subunit expressions were comparable between the control and PP2 treated SYF cells (Fig. 30). Combined, these results demonstrate the negative effects of c-Src on mitochondrial OXPHOS and energy metabolism, cell proliferation, and cell morphology which can be alleviated in the presence of an SFK inhibitor, such as PP2.

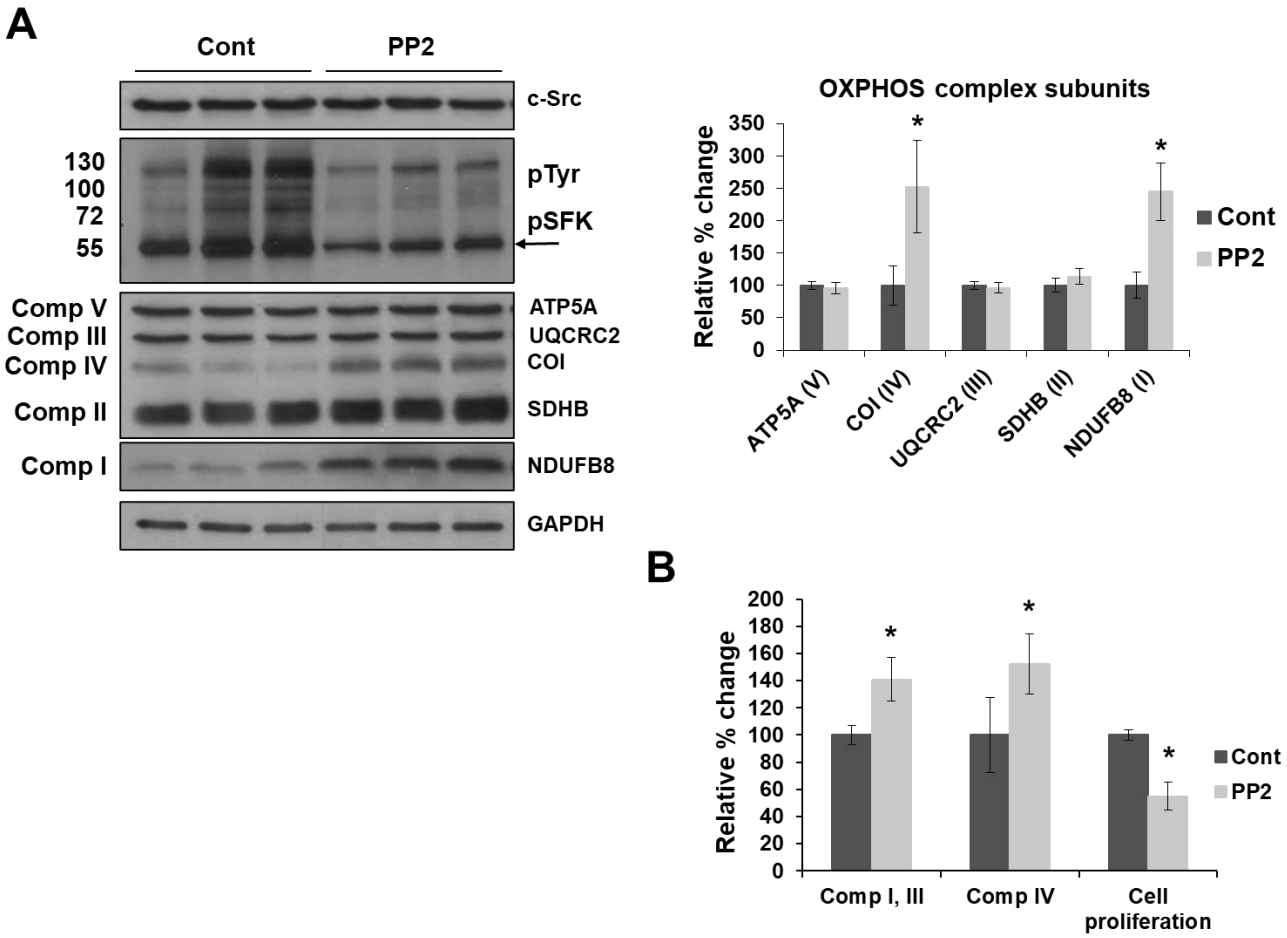


**Figure 28. Inhibition of c-Src stimulates mitochondrial OXPHOS expression in Src cells**. **A)** Src cells were treated with DMSO (cont) or 5 μM Src family kinase (SFK) inhibitor PP2 (PP2) for 24 h before collection. Changes in OXPHOS complex subunits, c-Src, and EF-Tu expression were measured by Western blot analyses with respect to GAPDH as a loading control. The phosphorylation of SFK members (pSFK), around 60 kDa, detected by the pTyr antibody, is indicated by an arrow. Quantitation of mitochondrial protein expression of OXPHOS complex subunits are represented as the mean ± SD of at least three experiments (right panel) and expressed as a percentage of the control cells (cont = 100%). Significant difference was observed between PP2 treated Src cells in mitochondrial-encoded COI and nuclear-encoded NDUFB8 subunits via an unpaired student's *t*-test (2-tailed), *P-value < 0.05. **B)** Cell proliferation, complex I and III activity, and complex IV enzymatic activity of PP2 treated Src cells were measured after 24 h treatments. Significant differences were observed between the cont and PP2 treated Src cells in the cell proliferation (P = 0.0003), complex I and III activity (P = 0.0001), and complex IV activity (P = 0.0001). Results are presented as the mean ± SD of at least three experiments and expressed as a percentage of the control cells (cont = 100%). The statistical significance was analyzed by unpaired student's *t*-tests (2-tailed), *P < 0.05. See Fig. 26 legend for details.

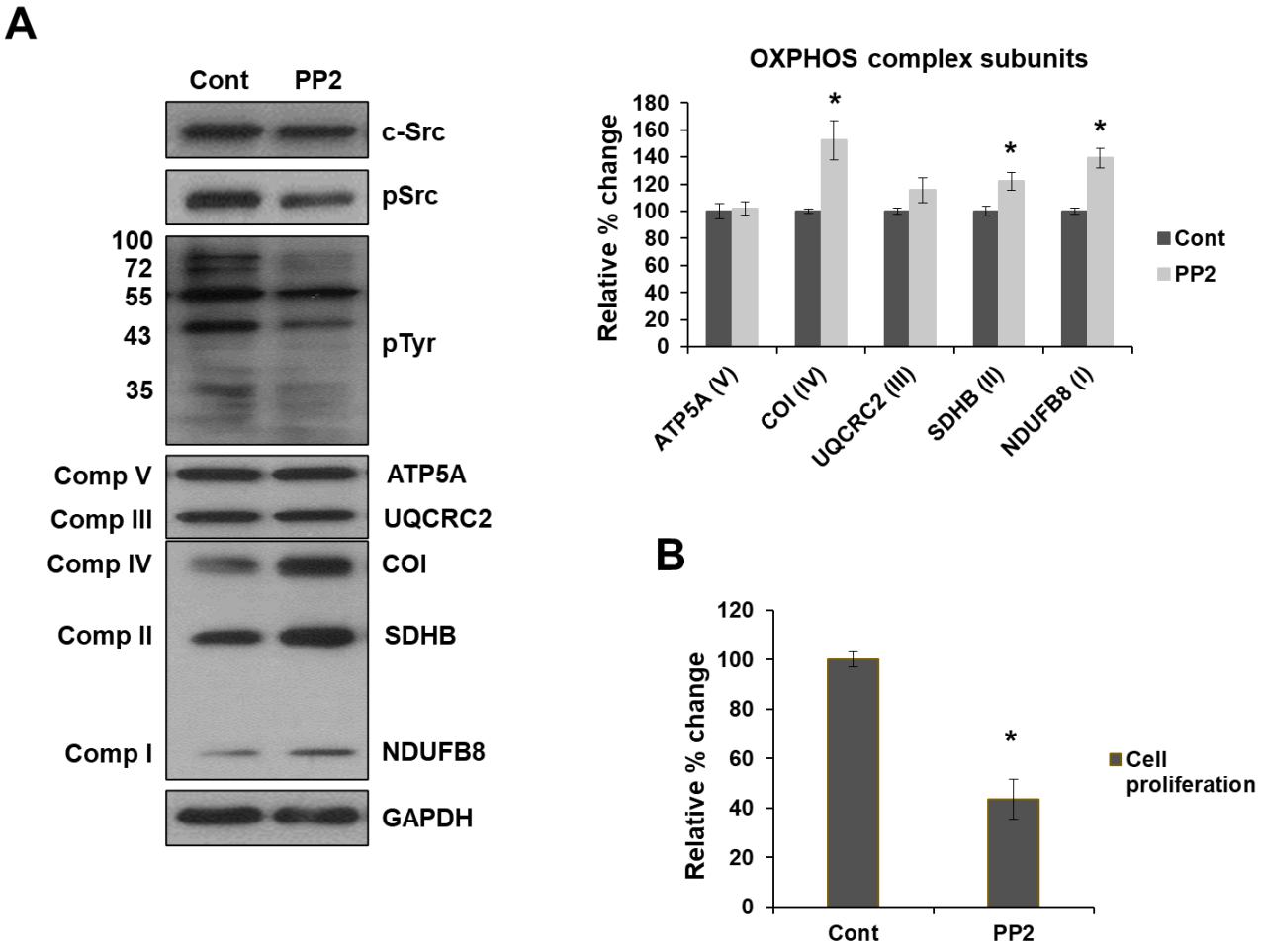


**Figure 29. Inhibition of c-Src with PP2 stimulates OXPHOS expression in Src$^{++}$ cells**. **A)** Src$^{++}$ cells were treated with 0 – 5 µM PP2 for 24 h before collection. The expression of OXPHOS complex subunits were detected by Western blot analyses with respect to GAPDH as a loading control. The quantitation of the protein expression of OXPHOS subunits are represented as the mean ± SD of at least three experiments (right panel) and are expressed as a percentage of the control cells (cont = 100%). Significant difference was observed between PP2 treated Src$^{++}$ cells in mitochondrial-encoded COI (P = 0.0084) and nuclear-encoded NDUFB8 (P = 0.0022) subunits. **B)** Cell proliferation was measured between the control and PP2 treated Src$^{++}$ cells and significant difference was observed (P = 0.0003). Results are presented as the mean ± SD of at least three experiments and expressed as a percentage of the control cells (cont = 100%). The statistical significance was analyzed by unpaired student's *t*-tests (2-tailed), *P < 0.05. See Fig. 28 legend for details.

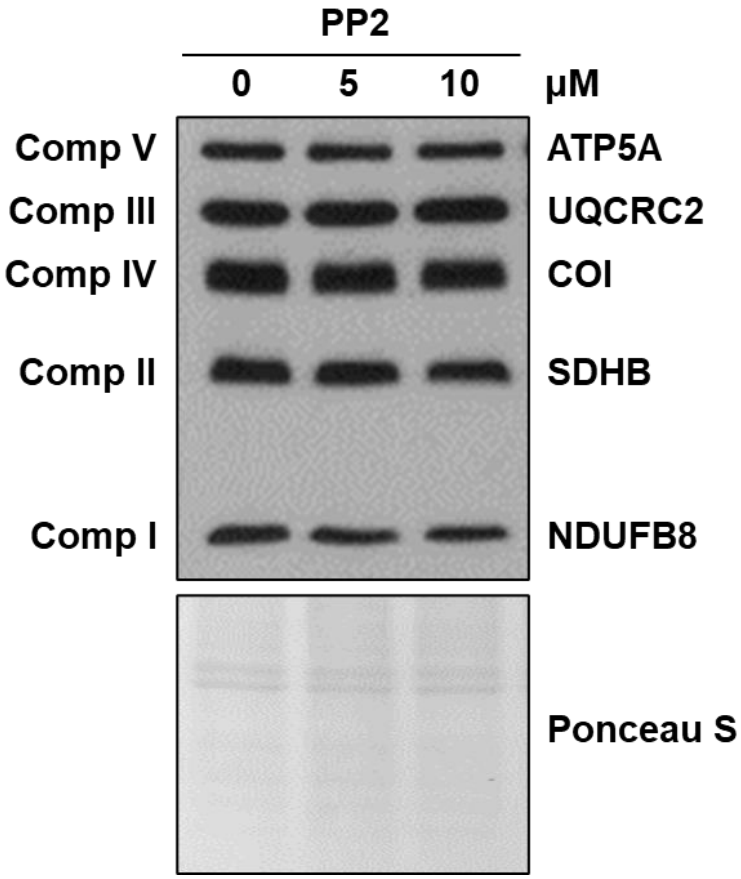


**Figure 30. PP2 treatments of SYF cells reduce cell proliferation without changing the expression of OXPHOS complexes**. SYF cells were treated with 0 – 10 μM PP2 for 48 h before collection. Expression of OXPHOS complex subunits were detected by Western blot analyses with respect to Ponceau S staining of the membrane. See Fig. 28 legend for details.

### 4.3.6. c-Src Knock Down Increases OXPHOS Complex Expression

Above, we demonstrated that the increasing expression of c-Src was correlated with a decrease in OXPHOS subunit expression and activity in cells treated with PP2. To further investigate c-Src inhibition, we transfected WT, $Src^{++}$, and Src cell lines with mouse specific c-Src siRNA. The c-Src expression was significantly reduced in WT, $Src^{++}$, and Src cell lines (Figs. 31A, 31B, and 31C, respectively) transfected with c-Src siRNA relative to cells treated with control siRNA. A significant increase was observed in the expression of NDUFB8 and COI of complexes I and IV, respectively, in all three cell lines transfected with c-Src siRNA (Fig. 31). The remainder of the OXPHOS complexes analyzed, were comparable between the cells transfected with the control and c-Src siRNAs. Clearly, the increases in OXPHOS complex expression was a result of the c-Src knock down in these MEF cell lines expressing c-Src to varying degrees. Therefore, our findings strongly suggest that c-Src regulates mitochondrial energy metabolism by inhibiting the expression and activity of OXPHOS complexes, specifically complexes I and IV. Furthermore, our results demonstrate that therapies targeting c-Src kinase alleviate the inhibition on mitochondrial oxidative phosphorylation and could be beneficial for the treatment of cancers with elevated c-Src expression and activity.

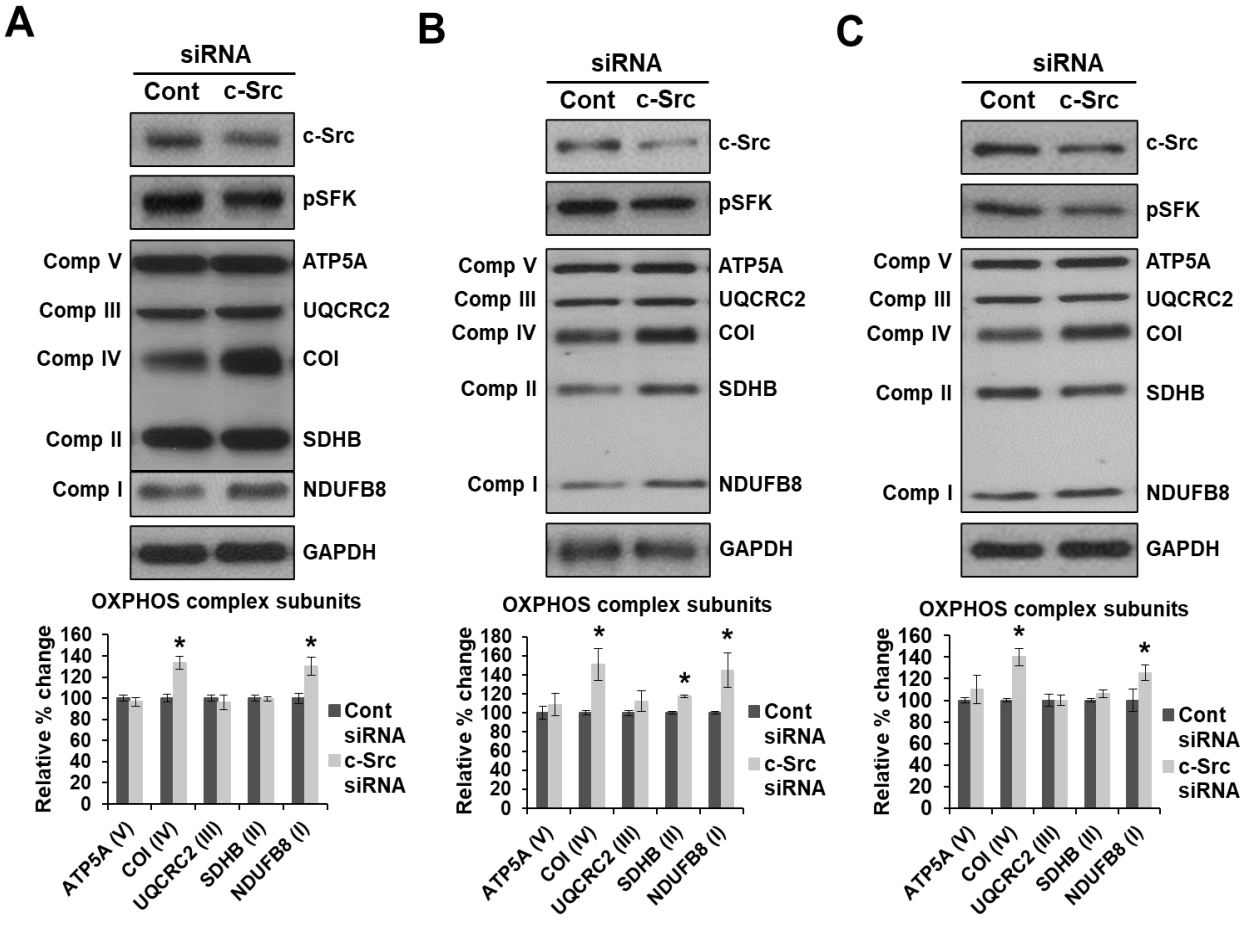


**Figure 31. c-Src knock down increases the expression of OXPHOS complexes**. **A)** WT, Src$^{++}$ **(B)**, and Src **(C)** cells were transfected with c-Src specific siRNA (c-Src) and control siRNA (cont). The expression of OXPHOS complex subunits were detected by Western blot analyses with respect to GAPDH antibody probing and Ponceau S membrane staining as loading controls. Quantitation of the expression of OXPHOS complex subunits are represented as the mean ± SD of at least three experiments (right panel) and expressed as a percentage of the control siRNA cells (cont = 100%). Significant difference was observed between c-Src and control siRNA transfected cells in the expression of NDUFB8 and COI in WT cells (P = 0.0001 and P = 0.0029, respectively), Src$^{++}$ cells (P = 0.0020 and P = 0.0056, respectively), and Src cells (P = 0.0004 and P = 0.0356, respectively). Statistical differences were analyzed between the cont and c-Src siRNA transfected cells with the unpaired student's *t*-tests (2-tailed), *P < 0.05.

## 4.4. Conclusions

Increased c-Src expression and activity are recognized as major underlying factors in the development of various diseases, including cancer, and is also suggested to contribute to increased metastatic, recurrence, and resistance rates in HCC (Masaki et al., 1998; R. Zhao et al., 2015). Although many systemic agents have been studied, to date, Sorafenib is the only approved drug for the systemic treatment of HCC, which has been shown to only extend the life of patients by 3-4 months (Daher et al., 2018; Llovet et al., 2008; Wilhelm et al., 2008). Interestingly, c-Src is located in the mitochondria and shown to alter mitochondrial energy metabolism via regulating OXPHOS complexes (Demory et al., 2009; Hebert-Chatelain et al., 2012; Miyazaki et al., 2003; Ogura et al., 2012). In some cases, c-Src has also been shown to induce the Warburg effect by inhibiting mitochondrial energy metabolism (Y. Jin et al., 2016; Wallace, 2012). Therefore, due to the various functions of c-Src in cell signaling, a better understanding of its role in mitochondrial energy metabolism in cancer and normal cells is essential.

In this study, we examined the effects of aberrant c-Src expression on mitochondrial energy metabolism by determining the relative changes in the expression of OXPHOS subunits in human liver cancer biopsies and cell lines. We clearly demonstrated that increased expression of c-Src was associated with an impaired expression of OXPHOS complex subunits in metastatic liver cancer biopsies and the Hep3B cell line. More importantly, we showed diminished OXPHOS complex expression and activity in mouse embryonic fibroblast cells expressing c-Src kinase alone. These results exhibited an inhibitory effect of c-Src on mitochondrial OXPHOS expression in both health and disease.

Both c-Src kinase and mitochondrial energy metabolism have become important targets for cancer therapy. We investigated the effects of c-Src inhibition on mitochondrial energy metabolism by treating cells with the SFK inhibitor PP2 as well as transfecting cells with c-Src siRNA. A significant increase in the expression of complex I and IV and their corresponding activities was shown in the metastatic Hep3B cell line in addition to c-Src expressing mouse fibroblast, WT, $Src^{++}$, and Src, cell lines treated with PP2 and c-Src siRNA. Interestingly, the inhibition of c-Src was also shown to reduce cell proliferation in Hep3B and c-Src expressing MEF cells, suggesting that c-Src regulates by modulating OXPHOS. Our results provide a better understanding of the relationship between c-Src and OXPHOS expression in normal and cancerous cells. Additionally, our results indicate that c-Src inhibition should be utilized in anti-cancer therapies to reduce cell proliferation and improve mitochondrial function.

### 4.5. Conflicts of Interest

The authors declare they have no conflict of interest.


### 4.6. Acknowledgements

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# CHAPTER 5

# FYN INHIBITION REDUCES THE EXPRESSION OF OXPHOS COMPLEXES AND CELL PROLIFERATION

Unpublished Manuscript in Preparation

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**Abstract**

Mitochondrial dysfunction plays a role in the development and progression of many different diseases, including cancer. The mitochondrion is not only important for energy production via oxidative phosphorylation (OXPHOS), but it also plays a major role in the production of reactive oxygen species (ROS), a byproduct of OXPHOS. Aberrant levels of ROS are major characteristics of mitochondrial dysfunction and the development and progression of various diseases and cancers. Fyn kinase, a Src family tyrosine (Tyr) kinase (SFK), is activated by oxidative stress and has been reported in various diseases. Additionally, Fyn was found in the mitochondria and shown to regulate mitochondrial OXPHOS. However, inhibitors that target both Fyn and mitochondrial OXPHOS have yet to be identified. In this study, we examined the role of Fyn kinase, along with the effect of the SFK inhibitor, SU6656, and natural antioxidants, kaempferol and resveratrol, on the expression of OXPHOS complexes. Fyn expression was correlated with high OXPHOS complex expression, which was reduced when cells were treated with SU6656 and kaempferol. Furthermore, the treatment of these cells with SU6656, kaempferol, and resveratrol significantly reduced cell proliferation. Evidence provided in this study reveals that Fyn regulates mitochondrial energy metabolism by altering the expression of OXPHOS complex subunits, which can be contributing factors to the development and progression of cancer. Our findings strongly suggest that the suppression of Fyn significantly reduces cell proliferation, implying that targeting the regulation of mitochondrial function by SFK inhibitors and natural antioxidants could prove valuable in cancer treatments.

## 5.1. Introduction

Mitochondria are unique organelles that are responsible for producing over 90% of the cell's energy in the form of ATP via oxidative phosphorylation (OXPHOS). The mitochondria also play essential roles in various cellular processes such as energy metabolism, cell survival and death, calcium homeostasis, and the generation of free radicals (Bhatti, Bhatti, & Reddy, 2017; J. A. Kim et al., 2008; Osellame et al., 2012). Reactive oxygen species (ROS), a family of free radicals (Halliwell, 2006), are produced as a byproduct of OXPHOS through leakage at complexes I and III (Q. Chen, Vazquez, Moghaddas, Hoppel, & Lesnefsky, 2003; Turrens, 2003; Turrens & Boveris, 1980). An uncontrolled production of ROS can contribute to oxidative stress which leads to cell damage, altered mitochondrial redox signaling, damage to regulatory proteins, mitochondrial membranes, and DNA, and mitochondrial dysfunction (Bhatti et al., 2017; Duchen, 2004; Finkel & Holbrook, 2000; Murphy, 2009). Interestingly, both mitochondrial dysfunction and oxidative stress have been reported as contributing factors in the development of a number of diseases including cancer (Bhatti et al., 2017; Dalle-Donne et al., 2006; Dhalla et al., 2000; Mitchell & Darley-Usmar, 2012; Petersen et al., 2003; Roberts & Sindhu, 2009; Sayre et al., 2001). Both genetic predisposition and environmental factors contribute to the development of diseases, which is attributed to oxidative stress generated through mitochondrial dysfunction. Although mitochondrial dysfunction has been established as a key factor in disease states, regulatory mechanisms for mitochondrial energy metabolism and ROS generation are not well understood.

Fyn kinase, a non-receptor Src family tyrosine (Tyr) kinase has emerged as a regulator of diverse pathological processes including the immune response, cell proliferation and adhesion, and has been shown to play a major role in metabolism and insulin signaling (Cary, Chang, &

Guan, 1996; T. W. Lee et al., 2013; Saito, Jensen, Salgia, & Posadas, 2010; Thomas & Brugge, 1997). Fyn was also reported as an inhibitor of AMP-activated protein kinase (AMPK) and fatty acid oxidation in the mitochondria (Bastie et al., 2007; Yamada, Pessin, Kurland, Schwartz, & Bastie, 2010). More importantly, Fyn is activated by oxidative stress and was located within the mitochondria where it regulates mitochondrial pathways (Lewandrowski et al., 2008; Salvi et al., 2002; Salvi et al., 2005). Recently, our laboratory has demonstrated the implication of Fyn with mitochondrial translation which is responsible for the synthesis of the 13 core subunits of OXPHOS complexes I, III, IV, and V and established the association of Fyn with increased mitochondrial translation and OXPHOS complex expression (E. C. Koc et al., 2016). As a result of its involvement in various cellular pathways, the hyperactivation of Fyn has contributed to the development of neurodegenerative diseases and multiple types of cancers (Elias et al., 2015; Saito et al., 2010; Schenone et al., 2011) indicating the need for novel therapeutic strategies. Although SFK inhibitors have been tested as potential cancer therapies (Finn et al., 2013; Lau et al., 2009; E. L. Mayer & Krop, 2010), the clinical data are limited. Due to the high levels of ROS found in cancer cells (Delmas et al., 2000; H. Guo et al., 2016; Liao et al., 2010; Zhu et al., 2018), natural antioxidants, kaempferol and resveratrol, have also been studied as chemotherapeutic agents. Even though these two antioxidants reduce cell proliferation in some studies and were reported to have inhibitory effects on Src kinase pathways (K. M. Lee et al., 2010; Oz et al., 2019), their role on the inhibition of Fyn and effects on mitochondrial OXPHOS remains to be elucidated.

In this study, we investigated the role of Fyn kinase on mitochondrial energy metabolism by analyzing the expression and activity of OXPHOS complexes. Additionally, we studied the effects of Fyn inhibition on mitochondrial energy metabolism and cell proliferation to examine

Fyn kinase as a potential target for the treatment of metabolic diseases with high Fyn kinase activity. Our studies strongly indicate that Fyn regulates the expression of OXPHOS complexes. Furthermore, our findings provide evidence that Fyn inhibition can be used as possible therapeutic strategies to prevent the progression of cancer by regulating mitochondrial energy metabolism and reducing cell proliferation.

## 5.2. Materials and Methods

### 5.2.1. Cell Culture

Monolayer cultures of HepG2 and Hep3B cell lines purchased from the American Type Culture Collection (ATCC) were maintained in DMEM medium (HyClone, Thermo-Scientific, Waltham, MA) adjusted to contain 4 mM glutamine, 1 mM pyruvate, 10% fetal bovine serum (FBS) (Rocky Mountain Biologicals, Missoula, MT), and 1% penicillin/streptomycin (P/S) (Corning Cellgro, Manassas, VA). The cells were grown in a humidified incubator at 37 °C and 5% $CO_2$. Collected cells were treated with sodium orthovanadate to preserve Tyr phosphorylation (Hebert Chatelain et al., 2011; Nishikawa et al., 2009).

For treatments, HepG2 and Hep3B cells were seeded at 25 x $10^4$ cells/mL in DMEM containing 0.2% FBS and incubated for 24 h. The media was then replaced with fresh DMEM containing 10% FBS and cells were treated for 48 – 72 h. The two cell lines were treated with the Src kinase inhibitor SU6656 **(**2,3-Dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide) (Millipore Sigma, St. Louis, MO) (Blake et al., 2000) dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 0 – 10 μM. Additionally, both cell lines were treated with natural antioxidants kaempferol and resveratrol (Calbiochem, EMD Millipore, Burington, MA), also dissolved in DMSO), at concentrations ranging from 0 – 50 μM, as previously described by our laboratory (Cimen et al., 2010). For cell

proliferation, cells were counted using the Trypan blue exclusion assay. The results are represented as the mean ± SD of triplicates from at least three experiments for each group and are presented as a percentage of the control (DMSO treated) cells.

### 5.2.2. Western Blot Analysis

Cell pellets were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, and protease and phosphatase inhibitor cocktails. Protein concentrations were determined by BCA assays (Pierce, Rockford, USA) using bovine serum albumin (BSA) as a standard. The protein lysates were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (Amersham, GE Healthcare, Pittsburg, PA) and stained with Ponceau S to ensure equal protein loading. The membranes were blocked in 5% (w/v) dry skim milk powder and 1% BSA dissolved in Tris-buffered saline (TBS) containing 0.05% Tween20 (TBST) and incubated with the corresponding primary antibodies overnight at 4 °C. The antibody for the OXPHOS complex cocktail was obtained from Mitosciences (Eugene, OR). The Fyn and phosphotyrosine (PY-20) antibodies were purchased from Sigma Aldrich (St. Louis, MO). The GAPDH antibody used as the protein loading control was obtained from Fitzgerald (Acton, MA). The phospho-Src family (pSFK) at Tyr416 (pTyr416) and phosphotyrosine (PY-100) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). The pSFK antibody detects the phosphorylation of Fyn and other family members, c-Src, Lyn, Yes, Hck, and Lck, at the kinase activation site, Tyr416, or the equivalent residue. Mitochondrial elongation factor Tu (EF-Tu) and mitochondrial transcription factor A (TFAM) antibodies were kind gifts from Dr. Linda Spremulli and Dr. Craig Cameron, respectively. The protein immunoreactivities were detected using the protocols provided by the manufacturer. Un-Scan-It (Silk Scientific Inc., Orem, UT) and ImageJ (Schneider et al., 2012)

were used to quantify protein band intensities. Quantified protein values were normalized to the protein loading determined by GAPDH antibody probing and Ponceau S staining of the membranes. The expression of each protein was quantified from individual cell lysates from at least three independent experiments. The results of the protein quantifications represent the mean ± SD for each sample and are expressed as a percentage of the control.

#### 5.2.3. Mitochondrial Complex IV Enzymatic Activity Assays

Cell pellets from HepG2 and Hep3B cell lines were dissolved in a 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 100 μM reduced cytochrome c. The assay was performed by monitoring cytochrome c oxidation at 550 nm, as previously described (Birch-Machin & Turnbull, 2001). The rate was calculated by dividing the difference in absorbances between two linear points by the difference in time points: Rate = (Abs 1 – Abs 2)/(Time 2 – Time 1). The enzymatic data are expressed as the mean ± SD of at least three experiments and are presented as a percentage of the control.

#### 5.2.4. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.07. Statistically significant differences were determined between HepG2 and Hep3B cell lines as well as control and treated cells using unpaired student's *t*-tests (2-tailed), $*P < 0.05$. All data are expressed as the mean ± SD, unless otherwise specified.

### 5.3. Results and Discussion

#### 5.3.1. Fyn Kinase is Expressed in HepG2 Cells and Associated with Increased OXPHOS

Fyn kinase is expressed in a variety of cell types and has therefore been identified as a factor in the involvement of numerous diseases (Bastie et al., 2007; Elias & Ditzel, 2015;

Matsushima et al., 2016; Saito et al., 2010). Additionally, Fyn kinase is one of the few SFK members found within the mitochondria (Lewandrowski et al., 2008; Salvi et al., 2002; Salvi et al., 2005) and has recently been shown to regulate mitochondrial protein synthesis and OXPHOS expression (E. C. Koc et al., 2016). We evaluated two human liver cancer cell lines to study the role of Fyn expression on mitochondrial OXPHOS expression and energy metabolism. An increased expression of Fyn kinase was found in HepG2 cells compared to almost no expression in the Hep3B cell line (Fig. 32A). Although Fyn kinase expression was absent in Hep3B cells, the phosphorylation of SFK members at the activation site, Tyr416 (pSFK), was comparable between the two cell lines (data not shown). Tyr416 is located within the activation loop of the kinase domain and is therefore responsible for the activity of Fyn and other SFK members when phosphorylated (T. Hunter, 1987; Thomas & Brugge, 1997). Since the phospho-Src family kinase (pSFK) antibody detects endogenous Fyn phosphorylation at Tyr416, as well as other family members at equivalent activation sites, these findings imply that other SFKs besides Fyn are active in Hep3B cells.

The differential endogenous expression of Fyn kinase between HepG2 and Hep3B cell lines allowed us to investigate its role on the expression of OXPHOS complexes. Reduced expression of OXPHOS subunits of complexes II, III, and V, as well as a significant decrease of approximately 80% in the expression of subunits of OXPHOS complexes I and IV, were associated with the absence of Fyn kinase in Hep3B cells relative to the expression of OXPHOS complexes found in HepG2 cells (Fig. 32A). To determine the effect of endogenous Fyn kinase expression on mitochondrial function, complex IV enzymatic activity assays were performed in the two cell lines. The activity of complex IV was significantly lower by roughly 40% in Hep3B cells (Fig. 32B). This impaired activity could be a result of the reduced expression of one of the

core subunits of complex IV, COII. Due to the decreased expression in one of the essential 13 mitochondrial-encoded proteins COII, we analyzed the expression of mitochondrial transcription factor A (TFAM) and mitochondrial elongation factor Tu (EF-Tu) to determine if changes in two of the main transcription and translation factors contributed to the decreased expression of COII. However, minor differences were observed in the expression of TFAM (data not shown) and EF-Tu (Fig. 33A). To further evaluate the reduction in COII, we performed quantitative RT-PCR analyses on several mitochondrial-encoded transcripts to determine if a defect is present in the expression of mitochondrial genes. The mRNA expression of COII, and other mitochondrial-encoded genes analyzed, were comparable between HepG2 and Hep3B cells (data not shown). Together, these results suggest that the reduction in mitochondrial-encoded COII was possibly a result of the absence of Fyn kinase expression and the associated reduction in Tyr phosphorylation in the Hep3B cell line. These findings are also in agreement with results previously published by our group (E. C. Koc et al., 2016) and imply that Fyn kinase regulates mitochondrial energy metabolism, possibly by increasing the expression of OXPHOS complexes.

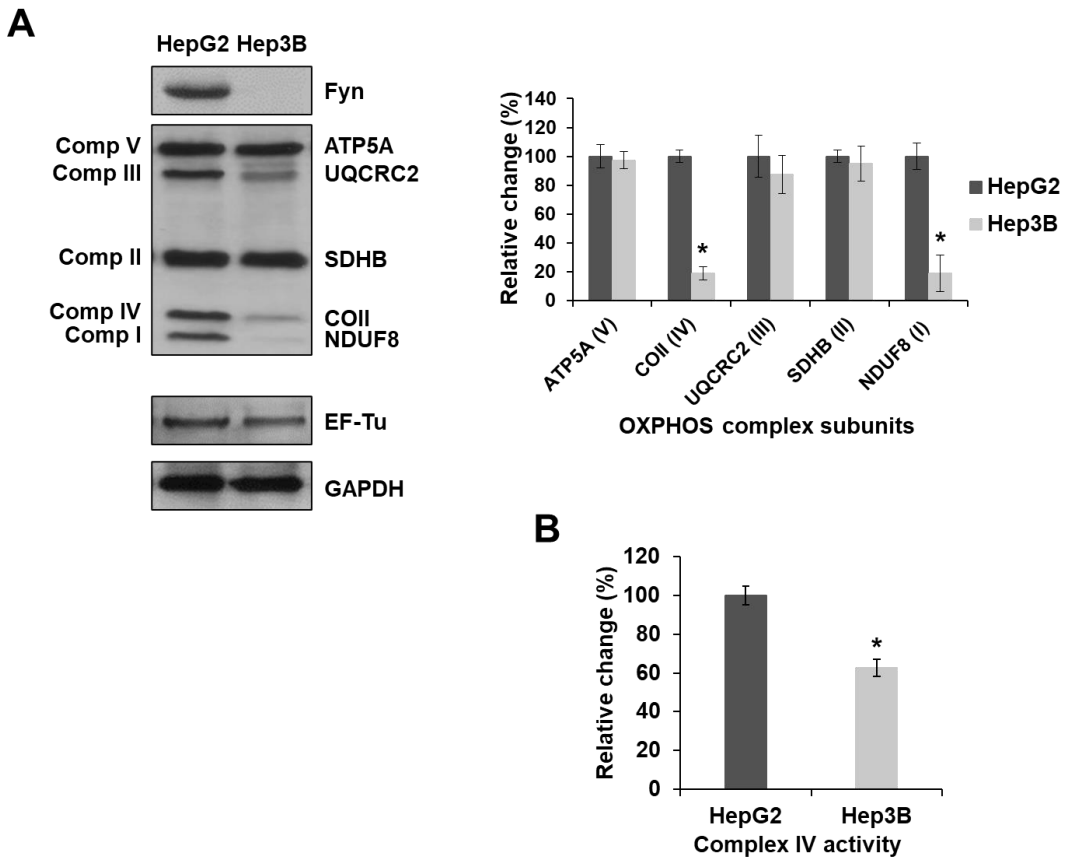


**Figure 32. Fyn increases OXPHOS expression in the HepG2 liver cancer cell line. A)** The relative expression of Fyn kinase, OXPHOS complex subunits, and mitochondrial proteins TFAM and EF-Tu were detected by Western blot analyses and compared between the HepG2 and Hep3B cell lines. The expression of OXPHOS subunits, including NDUF8 (complex I), SDHB (complex II), UQCRC2 (complex III), COII (complex IV), and ATP5A (complex V) were detected. Equal protein loading was determined by GAPDH antibody expression and Ponceau S staining of the membranes. Quantification of the average expression of OXPHOS complex subunits between the two cell lines is shown in the bar graph (right panel). The results represent the mean ± SD of at least three independent experiments. Significant difference was observed in OXPHOS subunits COII (P=0.001) and NDUF8 (0.0001) of complexes IV and I respectively between HepG2 and Hep3B cells. The protein quantitation data is presented as a percentage of HepG2 protein expression. **B)** Complex IV enzymatic activity was determined by spectrometically measuring the rate of cytochrome c oxidation at 550 nm using equal amounts of whole cell lysates from HepG2 and Hep3B cells. Enzymatic activity assay values are presented as mean ± SD for at least three experiments and presented as a percentage of the HepG2 complex IV activity. Significant difference was observed between HepG2 and Hep3B cells via unpaired student's *t*-test (2-tailed) with Welch's correction, *P < 0.05.

### 5.3.2. SU6656 Inhibits SFKs and Decreases OXPHOS Complex Expression

In an effort to further identify the role of Fyn in mitochondrial OXPHOS and energy metabolism, we treated HepG2 and Hep3B cell lines with the SFK inhibitor SU6656 and monitored the differences in the expression of OXPHOS complexes. SU6656 is a small molecule, potent inhibitor of ubiquitously expressed SFK members Fyn, c-Src, Yes, and Lyn (Blake et al., 2000). We first treated HepG2 cells with SU6656 and observed a decrease in Tyr phosphorylation with increasing concentrations of SU6656 (Fig. 33A). This reduction was associated with a decrease in the phosphorylated SFK band at Tyr416 (pSFK, pTyr416) implying that the SU6656 treatment inhibits the activation of SFKs in HepG2 cells (Fig. 33A). Although Tyr and SFK phosphorylation were reduced when cells were exposed to SU6656, the expression of Fyn was comparable to cells without treatment (Fig. 33A).

Subsequently, in addition to lower SFK and Tyr phosphorylation, reductions were observed in the expression of the mitochondrial-encoded subunit of complex IV and the nuclear-encoded subunit of complex II, COII and SDHB respectively, by approximately 25% with a more substantial reduction of roughly 45% found in the expression of complex I subunit NDUF8 at the highest SU6656 concentration (Fig. 33A). The expression of the remainder of OXPHOS subunits, UQCRC2 and ATP5A (Fig. 33A), along with EF-Tu and TFAM (data not shown) were comparable between the treated and control HepG2 cells. Our findings with SU6656 treatments are supported by previous studies by our laboratory demonstrating reduced mitochondrial translation with Fyn inhibition by siRNA (E. C. Koc et al., 2016), implying the effect of SU6656 treatment is a result of reduced Fyn kinase activity. Additionally, due to the role of Fyn activity on cell growth and proliferation (Cary et al., 1996; Schlessinger, 2000; Thomas & Brugge, 1997), we assessed the effect of Fyn inhibition by SU6656 on cell proliferation and observed a

significant reduction in HepG2 cell proliferation with increasing concentrations of SU6656 (Fig. 33B). Together our data further suggests that Fyn regulates mitochondrial energy metabolism by stimulating OXPHOS complex expression. Furthermore, treatment with SU6656 reduces OXPHOS expression as well as cell proliferation, implying a potential role of Fyn in cell growth in health and disease.

As a result of the impaired expression of OXPHOS complexes and the significant reduction in the proliferation of HepG2 cells treated with SU6656, we also treated the Hep3B cell line with SU6656 to observe possible changes in energy metabolism since it has a comparable expression of pSFK to HepG2 cells. Similar to HepG2 cells, lower SFK phosphorylation which coincided with reduced Tyr phosphorylation was found in Hep3B cells treated with SU6656, yet minor differences were observed in the expression of mitochondrial OXPHOS complexes (data not shown). Interestingly, a clear reduction in cell proliferation was shown in SU6656 treated Hep3B cells in a concentration-dependent manner (data not shown). The differences in the expression of OXPHOS complexes in SU6656 treated HepG2 and Hep3B cells are possibly due to the endogenous expression of Fyn kinase in HepG2 cells compared to almost no expression in Hep3B cells. These findings further imply that Fyn regulates mitochondrial energy metabolism by altering the expression of OXPHOS complexes and suggest that SFK inhibition can be beneficial for the treatment of diseases with high Fyn activity.

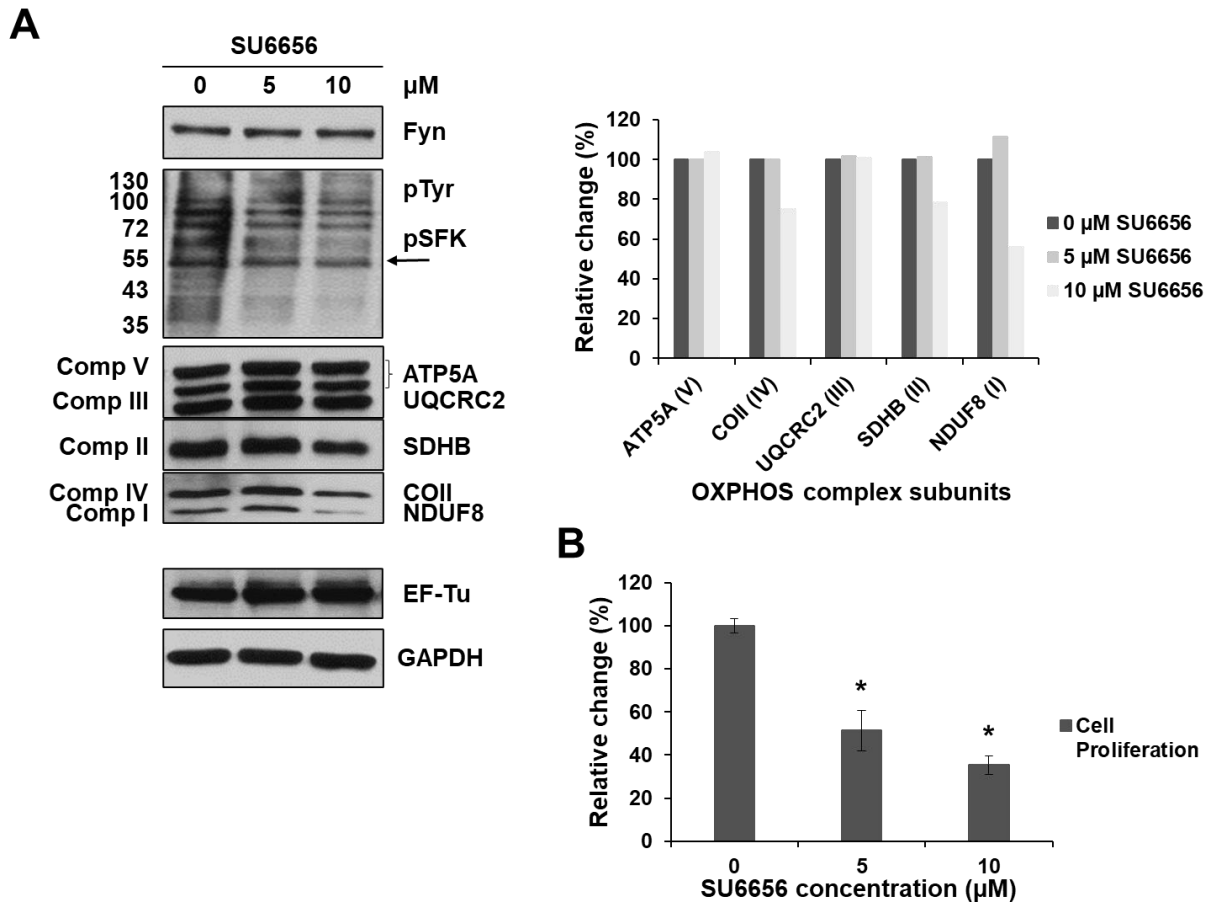


**Figure 33. SU6656 inhibits SFKs and decreases OXPHOS expression HepG2 cells.** HepG2 cells were treated with 0 – 10 µM SFK inhibitor SU6656 for 72 h before collection. **A)** The expression of OXPHOS complex subunits, Fyn, pTyr, EF-Tu, and TFAM were measured by Western blot analyses with respect to GAPDH antibody probing and Ponceau S staining of the membranes as controls for equal protein loading. The phosphorylation of SFKs (pSFK) was detected by the pTyr antibody and indicated by an arrow. The quantification of OXPHOS subunits is represented in bar graph (right panel). **B)** Cell proliferation of SU6656 treated HepG2 cells were measured with the Trypan blue exclusion assays after 72 h treatment and is shown as the mean ± SD of at least three experiments with statistical significance between the control and 5 µM (P=0.001) and 10 µM (P=0.001) SU6656. The results are represented as a percentage of the control. Statistical difference was observed between the control and treatment groups via unpaired Student's *t*-test (2-tailed), *P < 0.05.

### 5.3.3. Kaempferol Impairs OXPHOS Expression by Inhibiting SFKs

Kaempferol and resveratrol have anti-oxidant, chemo-preventative, anti-tumor, and anti-inflammatory pharmacological activities (H. Guo et al., 2016; Y. B. Huang et al., 2014; Liao et al., 2010; M. Singh et al., 2014; Udenigwe et al., 2008; Zhu et al., 2018) and have been shown to improve mitochondrial function in various cancer types (Cimen et al., 2010; Haohao et al., 2015; Lagouge et al., 2006). In addition to its many beneficial properties, kaempferol was shown to act as a c-Src kinase inhibitor by blocking the ATP-binding site of Src kinases and inhibiting their downstream signals (K. M. Lee et al., 2010). Fyn has been reported to be activated when exposed to oxidative stress. Since mitochondria produce the majority of ROS via oxidative phosphorylation to meet biosynthetic demands for cell proliferation (Wallace, 2012), we treated HepG2 and Hep3B cells with natural antioxidants kaempferol and resveratrol to observe their effects on Fyn kinase activity and OXPHOS complex expression.

A reduction in SFK phosphorylation was observed in a concentration-dependent manner with a decrease of approximately 35% at the highest kaempferol concentration (Fig. 34A). This decrease in SFK phosphorylation was associated with a reduction in the Tyr phosphorylation of proteins in HepG2 cells treated with kaempferol (Fig. 34A). Although kaempferol was identified as a c-Src kinase inhibitor (K. M. Lee et al., 2010), the high sequence conservation between Fyn and c-Src makes Fyn a likely target for kaempferol's inhibitory effect. Therefore, our results imply kaempferol acts as an SFK inhibitor in HepG2 cells and reduces the activity of SFKs by decreasing phosphorylation at the activation site, Tyr416 (pSFK).

Along with reduced SFK phosphorylation, a clear decrease was observed in the nuclear-encoded subunits of complexes I, NDUF8, and II, SDHA/SDHB, and the mitochondrial-encoded subunits of complex IV, COII, with increasing concentrations of kaempferol (Fig. 34A). The

expression of the remainder of the complex III, UQCRC2, and complex V, ATP5A, subunits analyzed were comparable to the control cells (Fig. 34A). To determine if the decrease in the expression of mitochondrial-encoded COII was due to changes in mitochondrial transcription and/or translation, we studied the mRNA expression of mitochondrial-encoded transcripts and the protein expression of TFAM and EF-Tu. Minor differences were found in the mRNA transcript expression (data not shown) and the expression of TFAM (data not shown) and EF-Tu (Fig. 34A), indicating the decreases in OXPHOS expression were a result of changes in Tyr phosphorylation by SFK inhibition. These results are in agreement with our SU6656 treatment data above. Our findings further demonstrate the possible regulation of mitochondrial OXPHOS by Fyn kinase. Furthermore, a significant reduction in cell proliferation was observed in a concentration-dependent manner (Fig. 34B). These findings reveal that kaempferol acts as an SFK inhibitor to reduce OXPHOS expression and lower cell proliferation. Therefore, kaempferol could potentially be used as a therapeutic tool to prevent the progression of cancer.

To observe if kaempferol had similar effects on Hep3B cells, the Hep3B cell line was also treated with kaempferol, which did not cause major changes in the expression of OXPHOS complexes under these conditions (Fig. 35A). Interestingly, a reduction in SFK phosphorylation, as shown with the pTyr antibody, and a correlated decrease in Tyr phosphorylation was found when Hep3B cells were treated with kaempferol (data not shown). However, similar to HepG2 cells, Hep3B cell proliferation was significantly decreased with increasing concentrations of kaempferol (Fig. 35B). Our findings were also supported by previous studies demonstrating that kaempferol-mediated reduction of the proliferation of Hep3B cells (Berger et al., 2013). Together, these results suggest that kaempferol acts as an SFK inhibitor in both cell lines and dramatically reduces cell proliferation.

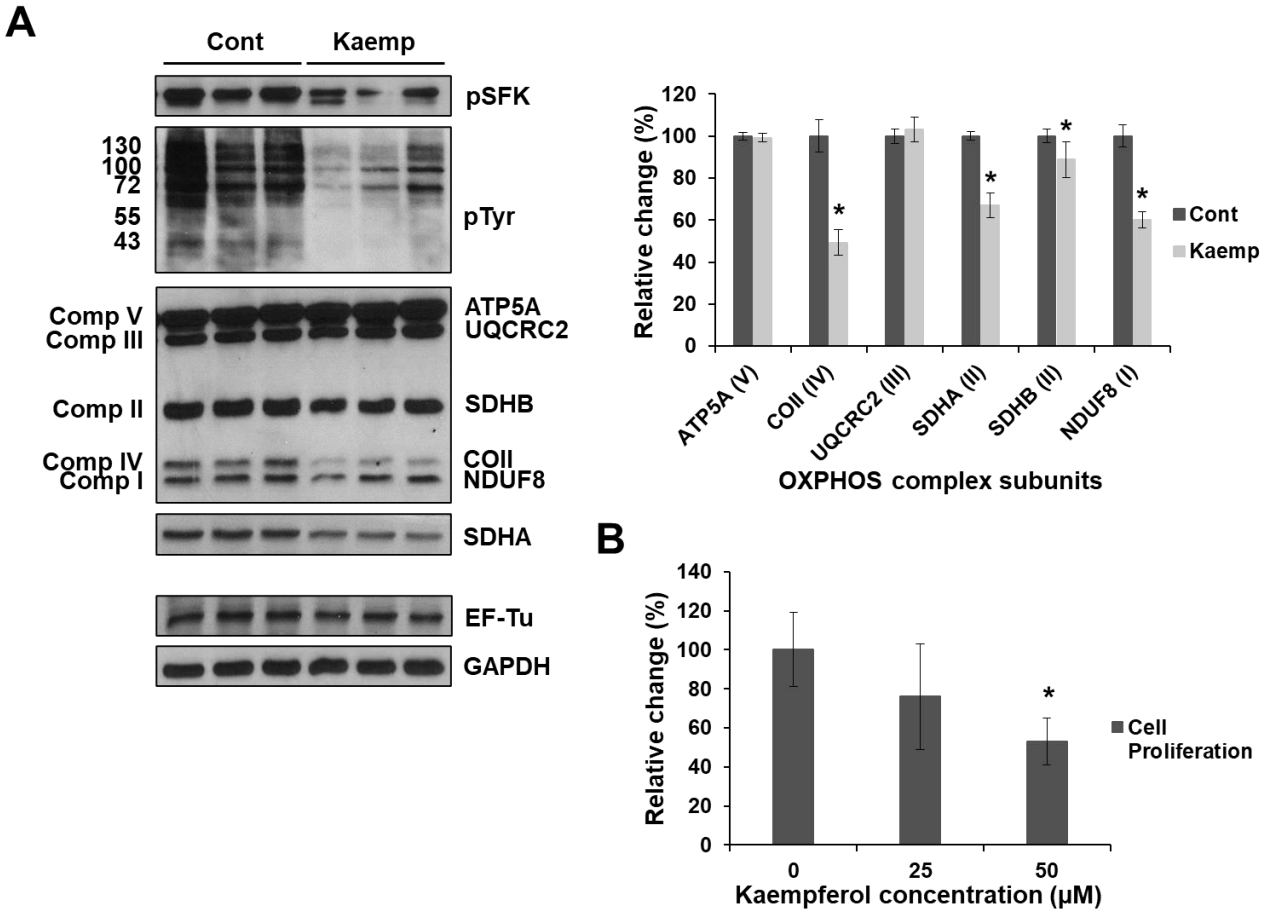


**Figure 34. Kaempferol impairs OXPHOS, reduces cell proliferation, and inhibits SFK activity in the HepG2 cell line.** HepG2 cells were treated with 0 – 50 µM kaempferol for 48 h before collection. **A)** The expression of OXPHOS subunits, phosphorylated Src at Tyr416 (pSFK), pTyr, EF-Tu, and TFAM were detected by Western blot analyses in three cell lysates with equal protein loading was evaluated by GAPDH antibody probing and Ponceau S staining of the membranes. The average quantitative analyses of the expression of OXPHOS subunits in the triplicates is shown in the bar graph (right panel) and statistical difference was observed in the expression of NDUF8 (P=0.001), SDHA (P=0.0007), SDHB (0.0135), and COII (0.0001) between the control and 50 µM kaempferol treatment (Kaemp). The results are represented as the mean ± SD of at least three experiments. **B)** Cell proliferation of kaempferol treated HepG2 cells was measured with the Trypan blue exclusion assays after 48 h treatment. Data is represented as the mean ± SD and statistical significance was found at 50 µM (P=0.0001) kaempferol. The results are represented as a percentage of the control. Statistical difference was observed between the control and treatment groups via unpaired Student's *t*-test (2-tailed), *P < 0.05.

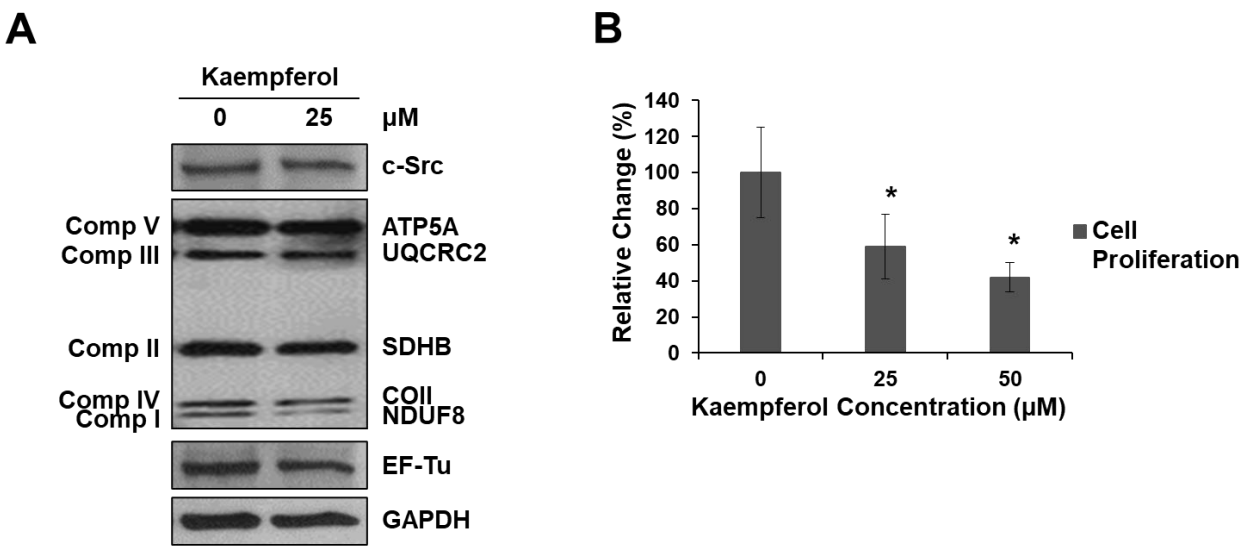


**Figure 35. Kaempferol suppresses cell proliferation in the Hep3B cell line.** Hep3B cells were treated with 0 – 50 μM kaempferol for 48 h before collection. **A)** The expression of OXPHOS complex subunits were measured by Western blot analyses with respect to GAPDH antibody probing and Ponceau S staining of the membranes as equal loading controls for the control (0) and 25 μM (25) kaempferol treatments. **B)** Cell proliferation of kaempferol treated Hep3B cells was measured with the Trypan blue exclusion assays after 48 h treatment. Statistical significance was observed between the control and 50 μM kaempferol treatment (P=0.0001). Results represent the mean ± SD of at least three experiments and are presented as a percentage of the control cells. Significant difference was analyzed by unpaired Student's *t*-tests (2-tailed), *P < 0.05. See Figure 34 legend for details.

### 5.3.4. Resveratrol Reduces Cell Proliferation

The natural antioxidant kaempferol showed promise as a potential therapeutic agent to improve cellular stress by decreasing OXPHOS expression in HepG2 cells and inhibiting cell proliferation in both HepG2 and Hep3B cell lines. To determine whether other natural antioxidants can be used as a treatment for metabolic disorders and mitochondrial dysfunction, we treated the two liver cancer cell lines with resveratrol for 48 h. In HepG2 cells, we found minor differences in the expression of nuclear- and mitochondrial-encoded OXPHOS subunits when treated with resveratrol (Fig. 36A); however, a significant decrease in the cell proliferation was observed (Fig. 36B). Similar results were obtained in Hep3B cells treated with resveratrol with the expression of OXPHOS subunits comparable between the control and treated cells (Fig. 37A) while the cell proliferation was substantially diminished in a concentration-dependent manner (Fig. 37B). Resveratrol has been shown to inhibit Src kinase pathways in cell cultures (Oz et al., 2019), yet its role as an SFK inhibitor and the effects on mitochondrial OXPHOS have not been identified. Under these conditions, resveratrol did not alter the steady-state expression of OXPHOS subunits, but it clearly decreased cell proliferation in both HepG2 and Hep3B cell lines, as demonstrated previously (Delmas et al., 2000; Udenigwe et al., 2008). Together, our findings suggest that SFK inhibitors and natural antioxidants kaempferol and resveratrol should be utilized in treatment regiments to prevent the progression of liver cancer by impairing cell proliferation.

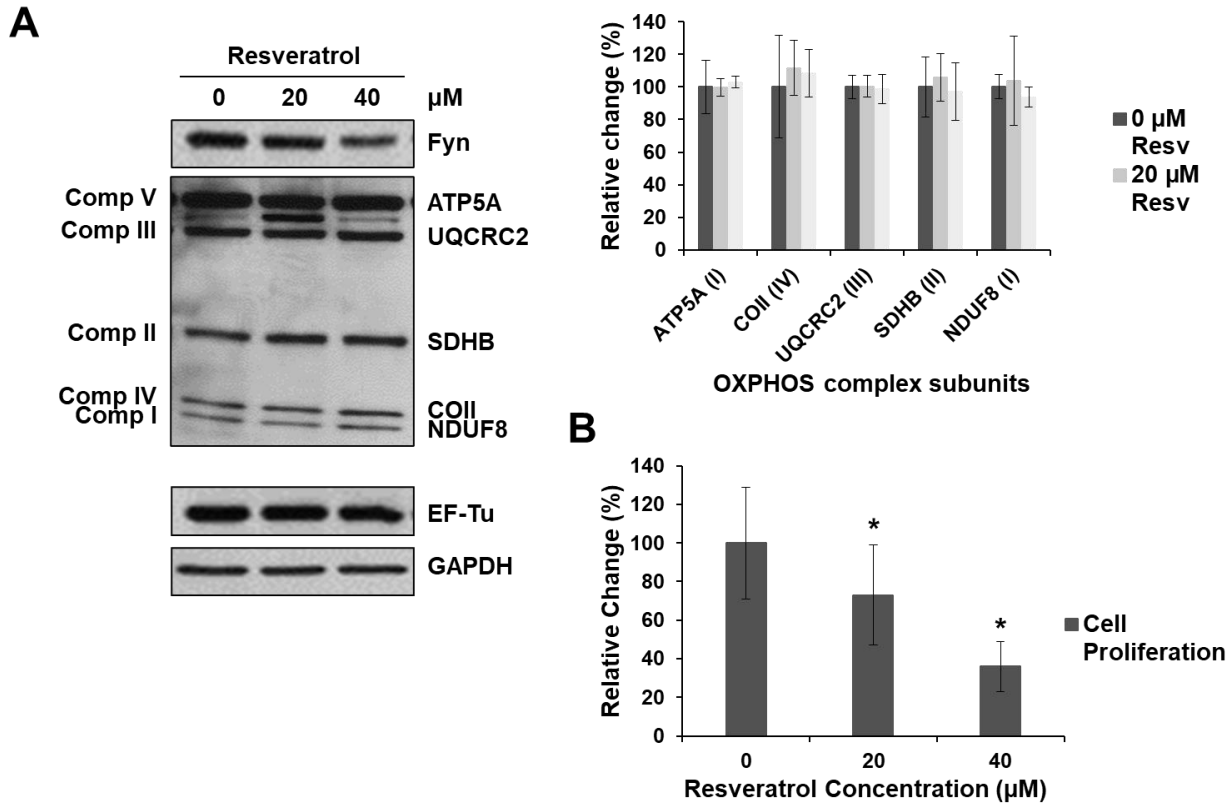


**Figure 36. Resveratrol treatment inhibits cell proliferation in the HepG2 cell line.** HepG2 cells were treated with 0 – 40 μM resveratrol for 48 h before collection. **A)** The expression of OXPHOS complex subunits were measured by Western blot analyses with respect to GAPDH antibody probing and Ponceau S staining of the membranes as equal loading controls. **B)** Cell proliferation of resveratrol treated HepG2 cells was measured with the Trypan blue exclusion assays after 48 h treatment. Significant reduction was observed at 20 μM (P=0.0269) and 40 μM (P=0.0089) resveratrol concentrations. Results represent the mean ± SD of at least three experiments and are presented as a percentage of the control (0). Statistical differences were analyzed by unpaired student's *t*-tests (2-tailed) for statistical significance, p-value < 0.05 (*).

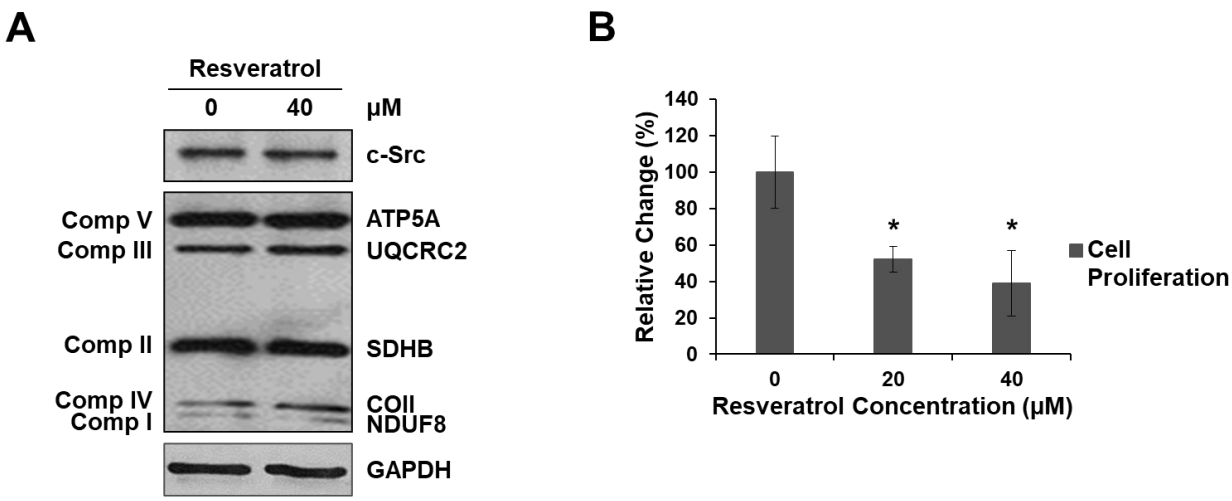


**Figure 37. Resveratrol impairs cell proliferation in the Hep3B liver cancer cell line.** Hep3B cells were treated with 0 – 40 μM resveratrol for 48 h before collection. **A)** The changes in OXPHOS complex subunit expression was measured by Western blot analyses with respect to GAPDH antibody probing and Ponceau S staining of the membranes as equal loading controls. **B)** Cell proliferation of resveratrol treated Hep3B cells was measured with the Trypan blue exclusion assays after 48 h treatment. Significant reduction was observed at both 20 μM (P=0.02) and 40 μM (P=0.0001) resveratrol treatments. The results represent the mean ± SD of at least three independent experiments and are presented as a percentage of the control (0). Statistical significance was analyzed between the control and treatment groups via unpaired Student's *t*-test (2-tailed), *P < 0.05. See Fig. 36 legend for details.

## 5.4. Conclusions

Mitochondria are essential for energy metabolism and ATP production via oxidative phosphorylation and are also major contributors to the generation of ROS. Uncontrolled ROS production can be detrimental to the cell and lead to mitochondrial dysfunction, which has been reported as a factor in the development and progression of numerous diseases including cancer (Bhatti et al., 2017; Dalle-Donne et al., 2006; Dhalla et al., 2000; Mitchell & Darley-Usmar, 2012; Petersen et al., 2003; Roberts & Sindhu, 2009; Sayre et al., 2001). Fyn kinase was located in the mitochondria, shown to regulate mitochondrial translation and OXPHOS, and activated by oxidative stress (E. C. Koc et al., 2016; Lewandrowski et al., 2008; Salvi et al., 2002; Salvi et al., 2005). Aberrant activation of Fyn contributed to the development of many diseases and has been reported as a key player in cancer resistance (Elias et al., 2015; Saito et al., 2010; Schenone et al., 2011), yet to date, no Fyn inhibitors are currently available. Therefore, understanding the role of Fyn and its inhibition on cell signaling and mitochondrial OXPHOS and energy metabolism is crucial for the treatment of metabolic diseases.

In this study, we demonstrated that Fyn kinase expression was associated with increased expression of OXPHOS complexes in liver cancer cell lines. These results suggested that the aberrant activation of Fyn could result in the continual stimulation of OXPHOS and mitochondrial dysfunction. To alleviate these effects and improve mitochondrial energy metabolism, we treated cells with the SFK inhibitor, SU6656, and observed decreased OXPHOS complex expression and cell proliferation. Additionally, we treated the cells with the natural antioxidants, kaempferol and resveratrol, and found a significant reduction in cell proliferation with both antioxidant treatments. A reduction in OXPHOS expression was only found in HepG2 cells treated with kaempferol. These findings suggest Src-dependent pathways promote cell

proliferation in the two cell lines. Our data clearly show that Fyn regulates mitochondrial energy metabolism by increasing OXPHOS complex expression, which can be alleviated when cells are treated with SFK inhibitors. More significantly, our results highlight the importance of Fyn inhibition in the treatment of cancer to slow disease progression and improve mitochondrial energy metabolism.

### 5.5. Conflicts of Interest

The authors declare no conflicts of interest.


### 5.6. Acknowledgements

The authors would like to thank Drs. Craig Cameron, Pennsylvania State University, and Linda Spremulli, UNC Chapel Hill, for their kind donations of mitochondrial TFAM and EF-Tu antibodies, respectively. We also acknowledge the Department of Biomedical research at Marshall University School of Medicine for their support.

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# CHAPTER 6

# CONCLUSIONS AND FUTURE DIRECTIONS

## 6.1. Conclusions

Mitochondria are the metabolic hub of the cell and therefore, are responsible for producing most of the cellular energy, ATP, through oxidative phosphorylation (OXPHOS) and activating numerous cellular pathways. In mammals, the majority of OXPHOS complex subunits are encoded in the nuclear genome and translocated into the mitochondria; however, 13 core enzymatic subunits are encoded in the mitochondrial genome and synthesized within the mitochondria by their own translation machinery. Changes in the expression of mitochondrial proteins, including OXPHOS complexes, can be detrimental to many cellular pathways and more importantly, mitochondrial biogenesis and energy metabolism. In fact, mitochondrial dysfunction impacts cellular and whole-body metabolism and has been identified as the cause for several metabolic diseases such as T2D and cancer. Therefore, a better understanding of key players involved in the regulation of mitochondrial functions in health and disease is essential. In these studies, we investigated mitochondrial biogenesis and energy metabolism by observing changes in OXPHOS expression and activity and evaluated potential targets and therapies in two major metabolic diseases, type 2 diabetes (T2D) and liver cancer. Together, our studies clearly demonstrated that changes in the expression of OXPHOS complexes can significantly impair mitochondrial energy metabolism and may contribute to the development of T2D and liver cancer.

### 6.1.1. Mitochondrial Biogenesis is Impaired in TALLYHO/Jng Mice

The TALLYHO/Jng (TH) mouse is a polygenic model for human obesity and T2D (J. H. Kim & Saxton, 2012; J. H. Kim et al., 2001). Gene mapping, whole genome sequencing, and

metabolic characterizations have been performed and altered energy balance has been reported (Franko et al., 2017; J. H. Kim et al., 2005; X. Mao et al., 2014; Parkman et al., 2017; Parkman et al., 2016); however, the mechanism is largely unknown. Additionally, the role of mitochondrial biogenesis and oxidative phosphorylation has yet to be identified in TH mice. In this study, we investigated mitochondrial biogenesis by analyzing the expression of nuclear- and mitochondrial-encoded translation machinery and OXPHOS complex subunits in mitochondrial rich liver and kidney tissues of TH and B6 mice. We observed a significant decrease in the expression of nuclear-encoded complex I subunits, NDUFS2 and NDUFB8, in the liver and kidney of TH mice. These decreases in the expression were correlated with reduced complex I and III enzymatic activity. Interestingly, a sequence variant was identified in the NDUFS2 subunit that may be related to the impaired expression and activity of complex I. We also found a significant decrease in the mitochondrial-encoded subunits of complex IV, COI and COII, which were correlated with diminished activity of complex IV. The expression of complex IV is required for the stability and assembly of complex I (Diaz et al., 2006); therefore, the reduced expression of the subunit of complex IV may contribute to the reduced complex I expression observed in TH mice.

The reduction in COI and COII expression in TH mice indicated impaired mitochondrial protein synthesis, since it is responsible for the synthesis of the 13 mitochondrial-encoded core OXPHOS subunits. Although the expression of translation machinery was comparable between TH and B6 mice, a sequence variant was identified in DARS2 which was predicted to alter the tRNA binding during protein synthesis and inhibit OXPHOS expression in TH mice. Other translation components, such as MRPL20 and other MRPs, were also reported to have sequence

variants that may influence the mitochondrial protein synthesis and OXPHOS. We identified factors that may contribute to the impaired mitochondrial biogenesis found in TH mice.

### 6.1.2. c-Src Impairs OXPHOS Expression and Activity

Aberrant expression of c-Src has been shown to induce malignant properties in various cancer types including increased resistance, metastasis, and recurrence in HCC (Blume-Jensen & Hunter, 2001; Irby & Yeatman, 2000, 2002; M. P. Kim et al., 2009; Masaki et al., 1998; Yeatman, 2004). c-Src is located within the mitochondria and regulates mitochondrial energy metabolism by phosphorylating enzymes in carbohydrate metabolism, pyruvate decarboxylation, TCA cycle, and more important, OXPHOS complexes (Augereau et al., 2005; Hebert-Chatelain et al., 2012; Y. Jin et al., 2016; Lewandrowski et al., 2008; Miyazaki et al., 2003; Salvi et al., 2007). Interestingly, c-Src was shown to induce the Warburg effect by impairing mitochondrial energy metabolism (Y. Jin et al., 2016; Wallace, 2012). Although c-Src is reported as a major contributing factor to the development of HCC, its role in mitochondrial energy metabolism in liver cancer is currently unknown. In this study, we investigated the role of c-Src on mitochondrial energy metabolism in liver cancer and MEF cell lines. We clearly demonstrated that reduced mitochondrial OXPHOS expression was correlated with an increased expression of c-Src in metastatic liver cancer tissues and the metastatic HCC cancer cell line, Hep3B. The decrease in OXPHOS expression was associated with impaired activity of OXPHOS complexes I and III and complex IV. More importantly, we observed reduced OXPHOS complex expression and activity in MEF cell lines expressing varying levels of c-Src kinase. Our data signifies an inhibitory effect of c-Src on mitochondrial OXPHOS expression in health and disease.

Since c-Src and mitochondrial energy metabolism are currently targets for cancer therapies, we treated liver cancer and MEF cell lines with the SFK inhibitor PP2 to investigate

the inhibition of c-Src on mitochondrial energy metabolism. A significant increase was observed in the expression of OXPHOS complexes I and IV and their activities with increased concentrations of PP2 in both cell types. Interestingly, treatments with PP2 reduced cell proliferation in Hep3B and MEF cells. Since PP2 inhibits multiple SFKs, we studied the direct effect of c-Src inhibition on mitochondrial OXPHOS by transfecting Hep3B and MEF cells with c-Src siRNA. Similar to PP2 treatments, cells transfected with c-Src siRNA showed increased expression of OXPHOS complexes I and IV. These results indicate that c-Src inhibition should be utilized as anti-cancer therapies to improve mitochondrial function while slowing cell growth and proliferation. More importantly, our results clearly show the role of c-Src in the regulation of energy metabolism and provide a better understanding of the relationship between c-Src and OXPHOS in normal and cancerous cells.

### 6.1.3. Fyn Inhibition Reduces OXPHOS Expression and Cell Proliferation

Mitochondria are not only important for energy production via oxidative phosphorylation (OXPHOS), but it also plays a major role in the production of reactive oxygen species (ROS), a byproduct of OXPHOS. Uncontrolled ROS generation can be detrimental to the cell and has been identified as a major characteristic of mitochondrial dysfunction (Bhatti et al., 2017; Dalle-Donne et al., 2006; Dhalla et al., 2000; Mitchell & Darley-Usmar, 2012; Petersen et al., 2003; Roberts & Sindhu, 2009; Sayre et al., 2001). Fyn kinase, a Src family tyrosine (Tyr) kinase (SFK), is activated by oxidative stress and is located within the mitochondria and shown to regulate protein synthesis and OXPHOS (E. C. Koc et al., 2016; Lewandrowski et al., 2008; Salvi et al., 2002; Salvi et al., 2005). Aberrant activation of Fyn contributed to the development of many metabolic diseases and has been reported as a key player in cancer resistance (Elias et al., 2015; Saito et al., 2010; Schenone et al., 2011). Inhibitors that target both Fyn and

mitochondrial energy metabolism and OXPHOS have yet to be identified. In this study, we examined the role of Fyn kinase, along with the effect of the SFK inhibitor, SU6656, and natural antioxidants, kaempferol and resveratrol, on the expression of OXPHOS complexes. Increased Fyn expression was correlated with high OXPHOS complex expression. These results suggested that the aberrant activation of Fyn could result in the continual stimulation of OXPHOS and lead to increased ROS production and mitochondrial dysfunction. The increase in OXPHOS was alleviated when cells were treated with SU6656 and kaempferol. Furthermore, the treatment of these cells with SU6656, kaempferol, and resveratrol significantly reduced cell proliferation. Evidence provided in this study reveals that Fyn regulates mitochondrial energy metabolism by altering the expression of OXPHOS complex subunits, which can be a contributing factor to the development and progression of metabolic diseases such as cancer. Additionally, these results suggest Src-dependent pathways promote cell proliferation in the two cell lines. Our findings strongly suggest that the suppression of Fyn significantly reduces cell proliferation, implying that improving mitochondrial function by SFK inhibitors and natural antioxidants could prove valuable in the treatment of metabolic diseases.

### 6.2. Future Directions

In chapter 3, we reported impaired mitochondrial biogenesis, protein synthesis and OXPHOS activity in TH mice. We identified sequence variants in the previously published whole genome database for TH mice NDUFS2, DARS2, and MRPL20 as possible candidates for reduced mitochondrial protein synthesis. To further examine the role of mitochondrial biogenesis and energy metabolism in TH mice, mitochondria should be isolated from fresh liver and kidney tissues of TH and B6 mice. Similar experiments should be performed to observe the expression of mitochondrial proteins and measure the activity of OXPHOS complexes in isolated

mitochondria instead of whole cell lysates. In addition to animal tissues, changes in mitochondrial biogenesis and OXPHOS can be observed in various cell lines treated with very high glucose concentrations to mimic hyperglycemic conditions found in insulin resistance and T2D. The changes in the expression of OXPHOS complexes, as well as other mitochondrial proteins involved in biogenesis, can be evaluated at different glucose concentrations. The enzymatic activities of mitochondrial OXPHOS complexes should also be measured. Additionally, site-directed mutagenesis should be used to create the major protein variants found in TH mice to express within the cell culture models. Changes in the expression of OXPHOS complexes and their enzymatic activities should be observed. By altering the sequences of NDUFS2, DARS2, and MRPL20, we can evaluate the effects of these sequence variants on mitochondrial biogenesis and energy metabolism, as well as determine any changes in the metabolic activity that reflect changes observed in the obesity and T2D phenotypes of TH mice. Resveratrol has been shown to activate mitochondrial biogenesis by altering the expression and activity of PGC-1α and has been shown to improve glucose tolerance and oxygen consumption (Lagouge et al., 2006). Therefore, the effects of resveratrol on glucose tolerance and mitochondrial energy metabolism should be investigated by treating the cell lines with varying concentrations of resveratrol and observing changes in the expression and activity of OXPHOS complexes. These studies would provide a better understanding of the factors involved in changes in mitochondrial biogenesis that may lead to mitochondrial dysfunction in obesity and T2D and could provide therapeutic targets for improving disease prognosis and treatments.

In chapter 4, we determined the role of c-Src in the regulation of mitochondrial OXPHOS in cancerous and normal cells. To better understand the relationship, further studies need to be performed to identify c-Src targets within the mitochondria. Since complex IV subunits COI and

COII are mitochondrial-encoded and are significantly reduced in the presence of c-Src and mitochondrial proteins were shown to be associated with Fyn kinase, another SFK member closely related to c-Src (E. C. Koc et al., 2016), the phosphorylation of MRPs by c-Src should be evaluated. *In vitro* phosphorylation assays should be performed with isolated c-Src kinase and cell lysates from MEF cells overexpressing c-Src kinase (Src cells). After identification of phosphorylation sites, site-directed mutagenesis can then be performed to alter the Tyr residue to mimic phosphorylation, which can then be transfected into cells overexpressing c-Src to examine the effects on OXPHOS expression and activity. By identifying c-Src targets within the mitochondria, we can get a better understanding of the effects c-Src has on more mitochondrial pathways than OXPHOS, which should provide more targeted therapy options. Additionally, the liver cancer cell lines Hep3B and HepG2, as well as the MEF cells with varying expressions of c-Src, should be treated with FDA-approved multi-kinase inhibitors to observe their effects on mitochondrial OXPHOS expression. These findings would allow us to better understand the effects of these inhibitors on c-Src and how they alter mitochondrial energy metabolism.

In chapter 5, we evaluated the association of Fyn kinase with OXPHOS expression and its effects when liver cancer cell lines were treated with the SFK inhibitor SU6656 and natural antioxidants kaempferol and resveratrol. To better determine the effects of SU6656 and kaempferol on mitochondrial oxidative energy metabolism, cells should be treated with the two compounds and changes in mitochondrial biogenesis should be investigated. First, OXPHOS complex enzymatic activity should be measured with the whole cell lysates obtained from liver cancer cell lines treated with the two compounds. Furthermore, changes in the expression of other mitochondrial components involved in mitochondrial biogenesis and OXPHOS regulation should be observed in whole cell lysates treated with SU6656 and kaempferol compared to the

control cells. Due to the association between increased Fyn and OXPHOS expression, mitochondrial biogenesis and OXPHOS should be further evaluated in additional cell lines with high Fyn expression. Once the correlation between Fyn and OXPHOS expression is identified, cell lines should be treated with SU6656 and kaempferol to determine if these treatments can potentially be used to inhibit cell growth and proliferation and improve mitochondrial function in other cancer types. Fyn was also shown to phosphorylate MRPs (E. C. Koc et al., 2016); therefore, identifying more Fyn targets within mitochondrial energy metabolism will be beneficial to better understand the role of Fyn kinase on mitochondrial OXPHOS. *In vitro* phosphorylation assays should be performed to identify Fyn-specific mitochondrial targets, which can then be altered with site-directed mutagenesis, and transfected into various cell lines with Fyn overexpression to determine the effects of the variants on Fyn activity and mitochondrial energy metabolism. The results from these phosphorylation studies should provide phosphorylation targets for further analyses and investigation.

Although our laboratory has clearly demonstrated the role of mitochondrial dysfunction by impaired OXPHOS expression in T2D and liver cancer, further studies are needed to better understand the involvement of the sequence variants identified in TH mice as well as c-Src and Fyn kinase in the regulation of mitochondrial energy metabolism in T2D and liver cancer, respectively.

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## APPENDIX A: INSTITUTUIONAL REVIEW BOARD APPROVAL

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

July 13, 2020

Caroline Hunter
903 Saddle Dr.
Salem, VA 24153

Dear Ms. Hunter:

This letter is in response to the submitted dissertation abstract entitled *"Dysfunctional Mitochondrial Biogenesis: A Potential Underlying Cause for Metabolic Diseases."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(e) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

## APPENDIX B: ABBREVIATIONS

5’-UTR…5’-untranslated region

A…adenine

AA…amino acid

aa-tRNA…aminoacyl-tRNA

ACOX1…acyl-coenzyme A oxidase 1

ADP…adenosine diphosphate

AKAP121/84…protein kinase A anchoring protein

AMPK…activated protein kinase

ANT1…ATP/ADP translocase 1

ANT2…ATP/ADP translocase 2

ARS2…amino acyl-tRNA synthetases

A-site…aminoacyl-site

ATCC…American Type Culture Collection

ATP…adenosine triphosphate

ATP5A…adenosine triphosphate subunit alpha

ATP6…adenosine triphosphate synthetase subunit 6

ATP8…adenosine triphosphate synthetase subunit 8

AUA…adenine, uracil, adenine

AUG…adenine, uracil, guanine

B6…C57BL/6J mice

BCA…bicinchoninic acid

BCL-xL…B-cell lymphoma-extra large

BEZ…bezafibrate

BSA…bovine serum albumin

$Ca^{2+}$…calcium

CAC…citric acid cycle

cDNA…complementary deoxyribonucleic acid

$CO_2$…carbon dioxide

CO…cytochrome c oxidase

COBRE ACCORD…Center of Biomedical Research Excellence named Appalachian Center for Cellular transport in Obesity Related Disorders

COI…cytochrome c oxidase subunit I

COII…cytochrome c oxidase subunit II

COIII…cytochrome c oxidase subunit III

Cont…control

CRIF1…CR6-interacting factor 1

CS…citrate synthase

Csk…C-terminal Src kinase

Cys (C)…cysteine

Cys245…cysteine 245

Cys487…cysteine 487

Cyt…cytochrome

Cyt b…cytochrome b

DARS2…aspartyl-tRNA synthetase 2

*Dars2*…aspartyl-tRNA synthetase 2 gene

D-loop…displacement loop

DMEM…dulbecco's modified eagle's medium

DMSO…dimethyl sulfoxide

DNA…deoxyribonucleic acid

DOC…sodium deoxycholate

Dok4…downstream of tyrosine kinase docking protein 4

ECL…enhanced chemiluminescence

EDTA…ethylenediaminetetraacetic acid

EF-Tu…mitochondrial elongation factor Tu

EGFR…epidermal growth factor receptor

EGTA…ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EMT…epithelial mesenchymal transition

FATP…fatty acid transport protein

FBS…fetal bovine serum

FDA…U.S. Food and Drug Administration

fMet-tRNA…formylmethionine-tRNA

FOXO1…forkhead box O1

FH…fumarate hydratase

GABC…Genomics and Bioinformatics Core

GAPDH…glyceraldehyde 3-phosphate dehydrogenase

GDP…guanosine diphosphate

GLUT4…glucose transporter 4

GTP…guanosine triphosphate

G…guanine

$H_2O$…water

$H_2O_2$…hydrogen peroxide

H-strand…heavy strand

HB…hepatoblastoma

HCC…hepatocellular carcinoma

HCl…hydrochloride

Hep3B…human hepatocellular carcinoma cell line 3B

HepG2…human hepatocellular carcinoma cell line G2

HER2…human epidermal growth factor receptor 2

HGF…hepatocyte growth factor

HK1…hexokinase 1

HK2…hexokinase 2

HSP1…H-strand promoter 1

HSP2…H-strand promoter 2

HSP60…heat shock protein 60

IDH1…isocitrate dehydrogenase 1

IDH2…isocitrate dehydrogenase 2

IGF-1R…insulin-like growth factor-1 receptor

IL-8…interleukin-8

IM…inner membrane

IMS…inter membrane space

IRS1…insulin receptor substrate 1

IRS2…insulin receptor substrate 2

$K^+$…potassium

Kaemp…kaempferol

kDa…kiloDalton

L-strand…light-strand

LDHA…lactate dehydrogenase A

Leu…leucine

LSP…light-strand promoter

LSU…large subunit

MEF…mouse embryonic fibroblast

Met…methionine

Mfn2…mitofusin 2

MM…mitochondrial matrix

MMPs…matrix metalloproteinases

MTERF1…mitochondrial termination factor 1

mRNA…messenger ribonucleic acid

MRP…mitochondrial ribosomal protein

MRPL…mitochondrial ribosomal proteins of the large subunit

MRPL11…mitochondrial ribosomal protein of the large subunit 11

MRPL15…mitochondrial ribosomal protein of the large subunit 15

MRPS…mitochondrial ribosomal proteins of the small subunit

MRPS18-B…mitochondrial ribosomal protein of the small subunit 18-B

MRPS29…mitochondrial ribosomal protein of the small subunit 29

MRPS30…mitochondrial ribosomal protein of the small subunit 30

mt…mitochondria

mtDNA…mitochondrial deoxyribonucleic acid

mtEF-G1…mitochondrial elongation factor G1

mtEF-Ts…mitochondrial elongation factor Ts

mtEF-Tu…mitochondrial elongation factor Tu

mtEF-Tu:GTP:aa-tRNA…mitochondrial elongation factor Tu, GTP, and aa-tRNA complex

mtIF2…mitochondrial initiation factor 2

mtIF3…mitochondrial initiation factor 3

mtPEPCK…mitochondrial phosphoenolpyruvate carboxykinase

mtRF1a…mitochondrial translation release factor 1a

mtRRF1…mitochondrial ribosome recycling factor 1

mtRRF2…mitochondrial ribosome recycling factor 2

mTOR…mammalian target of rapamycin

N…normal tissue

NaCl…sodium chloride

NADH…nicotinamide adenine dinucleotide plus hydrogen

NASA…National Aeronautics and Space Administration

NCBI…National Center for Biotechnology Information

ND…NADH dehydrogenase

ND1…NADH dehydrogenase core subunit 1

ND2…NADH dehydrogenase core subunit 2

ND3…NADH dehydrogenase core subunit 3

ND4…NADH dehydrogenase core subunit 4

ND4L…NADH dehydrogenase core subunit 4L

ND5…NADH dehydrogenase core subunit 5

ND6…NADH dehydrogenase core subunit 6

NDUFA4…NADH:ubiquinone oxidoreductase 1 alpha subcomplex 4

NDUFA13…NADH:ubiquinone oxidoreductase subunit A13

NDUFB8…NADH:ubiquinone oxidoreductase subunit B8

NDUFB10…NADH:ubiquinone oxidoreductase subunit B10

NDUFS2…NADH:ubiquinone oxidoreductase core subunit 2

*Ndufs2*… NADH:ubiquinone oxidoreductase core subunit 2 gene

NDUFS3…NADH:ubiquinone oxidoreductase core subunit A13

NDUFV1…NADH:ubiquinone oxidoreductase core subunit V1

NDUFV2…NADH:ubiquinone oxidoreductase flavoprotein 2

NIH…National Institute of Health

NP40…nonyl phenoxypolyethoxylethanol

NRF-1… nuclear respiratory factor-1

NRF-2…nuclear respiratory factor-2

OM…outer membrane

OPA1…optic atrophy 1

OXPHOS…oxidative phosphorylation

P/S…penicillin/streptomycin

PDGFR…platelet-derived growth factor receptor

PDH…pyruvate dehydrogenase

PDHK1…pyruvate dehydrogenase kinase 1

PEP…phosphoenolpyruvate phosphatase

PGC-1α… peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGC-1β... peroxisome proliferator-activated receptor gamma coactivator 1-beta

PKA…protein kinase A

PMSF…phenylmethylsulfonyl fluoride

POLRMT…mitochondrial DNA-dependent RNA polymerase

PP2…(4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine)

pSFK…phosphorylated Src Family Kinases at Tyr416

PTEN…phosphatase and tensin homolog

PTMs…post-translational modifications

PTPα…protein tyrosine phosphatase alpha

PTCD1… pentatricopeptide repeat domain 1

PTPD1…protein tyrosine phosphatase D1

pTyr416…phosphorylated tyrosine 416

pTyr527…phosphorylated tyrosine 527

PY-20…phosphotyrosine antibody

PY-100…phosphotyrosine antibody

pYEEI…phosphotyrosine motif, phosphor Tyrosine-Glutamic acid-Glutamic acid-Isoleucine

qRT-PCR…quantitative real-time polymerase chain reaction

QTLs…quantitative trait loci

RIPA…radioimmunoprecipitation assay

RNA…ribonucleic acid

ROS…reactive oxygen species

rRNA…ribosomal ribonucleic acid

RSV…Rous sarcoma virus

RTK…receptor tyrosine kinase

SAT…subcutaneous adipose tissue

SD…standard deviation

SDH…succinate dehydrogenase

SDHA…succinate dehydrogenase complex flavoprotein subunit A

SDHB…succinate dehydrogenase complex flavoprotein subunit B

SDHC…succinate dehydrogenase complex flavoprotein subunit C

*Sdhc*…succinate dehydrogenase complex subunit c gene

SDHD…succinate dehydrogenase complex flavoprotein subunit D

SDS…sodium dodecyl sulfate

SDS-PAGE…sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser…serine

SFK…Src family kinases

SH…Src homology

SH1…Src homology domain 1, protein kinase domain

SH2…Src homology domain 2

SH3…Src homology domain 3

SH4…Src homology domain 4

SHP1…Src homology 2 domain-containing protein tyrosine phosphatase 1

SHP2…Src homology 2 domain-containing protein tyrosine phosphatase 2

SIFT…sorting intolerant from tolerant

siRNA…small interfering ribonucleic acid

SRA…Sequence Read Archive

Src cells…MEF cells with null mutations in Yes and Fyn, overexpressing c-Src

$Src^{++}$ cells…MEF cells with null mutations in Yes and Fyn, endogenous expression of c-Src

SSU…small subunit

Stat3…signal transducer and activator of transcription 3

SU6656…(2,3-Dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]

1H-indole-5-sulfonamide)

SYF cells…MEF cells with functional null mutations in Src, Yes, and Fyn

T…primary tumor

T2D…type 2 diabetes

TBS…Tris-buffered saline

TBST…Tris-buffered saline with Tween-20

TCA…tricarboxylic acid

TEFM…mitochondrial transcription elongation factor

TFAM…mitochondrial transcription factor A

TFB2M…mitochondrial transcription factor B2

TH…TALLYHO/Jng mice

Thr…threonine

Tris-HCl…Tris-hydrochloride

tRNA…transfer ribonucleic acid

Trp…tryptophan

TUFM…Tu translation elongation factor, mitochondrial

Tyr…tyrosine

Tyr416…tyrosine 416

Tyr527…tyrosine 527

UAA… uracil, adenine, adenine

UGA…uracil, guanine, adenine

UQCRC2…ubiquinol-cytochrome c reductase core protein 2

VDAC…voltage-dependent anion channel

VEGF…vascular endothelial growth factor

WT cells…wild-type MEF cells with endogenous expression of all SFKs

WV…West Virginia

WV-INBRE…West Virginia IDeA Network of Biomedical Research Excellence

## APPENDIX C: CURRICULUM VITAE

# Caroline Hunter

**Phone:** 540-589-8280
**Email:** cahunter589@gmail.com
**Address:** 903 Saddle Drive
Salem, VA 24153

## EDUCATION

**Marshall University** – Huntington, WV
- Biomedical Sciences (Research) Graduate Program
- Ph.D. Candidate – Cardiovascular, Obesity, and Diabetes Research

**Roanoke College** – Salem, VA
- Bachelor of Science, Biochemistry

## RESEARCH EXPERIENCE

**Graduate Researcher** – Huntington, WV — May 2013 – Present
- Department of Biomedical Research, Koc Laboratory

**Piedmont Eye Center** – Lynchburg, VA — Jan. 2012 – Aug. 2012
- Department of Clinical Research
- Data Specialist

**Student Researcher** – Salem, VA — May 2011 – Dec. 2011
- Roanoke College, Department of Chemistry
- Sarisky Laboratory

**Student Researcher** – Salem, VA — Sept. 2010 – April 2011
- Roanoke College, Department of Chemistry
- Steehler Laboratory

## LABORATORY SKILLS

**Experimental Techniques**
- Cell/ Tissue Culture
- Gene Silencing/Overexpression
- End point and real-time PCR
- PCR Primer Design
- DNA/RNA/Protein Extraction
- Western Blot Analysis
- Various Toxicity Assays
- Cell Viability Assays
- Protein Immunoprecipitation
- Enzymatic Assays
- Wes automated protein analysis system

- Mitochondrial OXPHOS complex activity assays
- Bacterial Cultures
- Molecular Cloning
- Site-directed Mutagenesis
- Protein Expression and Purification

**Software**

- Proficient in Microsoft Office, Adobe Design, ImageJ, UN-Scan-IT, and GraphPad Prism

## HONORS AND AWARDS

2017 **Outstanding Poster Presentation Award**

- Appalachian Regional Cell Conference
- Best Poster Presentation for Basic Science Category

2015 **Travel Award**

- Post-Doctorate/Graduate Travel Award from American Society of Biochemistry and Molecular Biology (ASBMB)

2015 **Selected for an Oral Presentation**

- Marshall University School of Medicine Research Day

2014 **Selected for an Oral Presentation**

- Appalachian Regional Cell Conference

2014 **Best Presentation Award**

- Marshall University School of Medicine Research Day
- Best Poster Presentation for Basic Science Category

2013 **Second Place Poster Presentation Award**

- Appalachian Regional Cell Conference
- Second Place for Poster Presentation for Basic Science Category

## FUNDING

**Graduate Research Fellowship Award** 2015

- NASA West Virginia Space Grant Consortium

## PROFESSIONAL AFFILIATIONS

- American Society of Biochemistry and Molecular Biology (ASBMB)
- American Chemical Society (ACS)
- American Association for Advancement of Science (AAAS)

## PUBLICATIONS

Lawag AA, Napper JM, **Hunter CA**, Bacon NA, Deskins S, El-Hmdani M, Govender SL, Koc EC, Sollars VE. 2017 HSP90 Inhibition and Cellular Stress Elicits Phenotypic Plasticity in Hematopoietic Differentiation. Cell Reprogram. Oct; 19(5):311-323. Doi: 10.1089/cell.2017.0001.

**Hunter CA**, Kartal F, Koc ZC, Murphy T, Kim JH, Denvir J, Koc EC. 2019 Mitochondrial oxidative phosphorylation is impaired in TALLYHO mice, a new obesity and type 2 diabetes animal model. Int J Biochem Cell Biol. Nov; 116 Doi: 10.1016/j.biocel.2019.105616

**Hunter CA**, Koc H, Koc EC. 2020. c-Src kinase impairs the expression of mitochondrial OXPHOS complexes in liver cancer. Cell Signal, 72, p. 109651. doi:10.1016/j.cellsig.2020.109651. Epub 2020 April 23.

**Hunter CA**, Plymale NI, Smee KM, Sarisky CA. 2019. Experimental characterization of two archaeal inosine 5'-monophosphate cyclohydrolases. PLOS One. Oct. https://doi.org/10.1371/journal.pone.0223983

**Hunter CA**, Koc H, Koc EC. Fyn kinase inhibition impairs mitochondrial energy metabolism and reduces cellular proliferation. *In Preparation*

## PRESENTATIONS

**Hunter, C.A.,** Kartal F, Koc ZC, Murphy T, Kim JH, Denvir J, Koc EC. (**November 2017**). Mitochondrial oxidative metabolism is impaired in TALLYHO mice, a new obesity and type 2 diabetes animal model. ARCC. Athens, OH. **Basic Sciences Poster Presentation Award (1st).**

**Hunter, C.A.**, Koc, H. and Koc, E.C. (**August 2017**). Mitochondrial oxidative metabolism is impaired in TALLYHO mice, a new obesity and type 2 diabetes animal model. JCESOM Research Day. Huntington, WV.

**Hunter, C.A.,** Kim, JK, Koc, H and Koc, E.C. (**June 2017**). Regulation of mitochondrial biogenesis in obesity and type 2 diabetes animal model. UMDF. Washington D.C.

**Hunter, C.A.**, Koc, H. and Koc, E.C. (**August 2016**). Role of antioxidants in kinase-dependent regulation of mitochondrial energy metabolism. JCESOM Research Day. Huntington, WV.

**Hunter, C.A.**, Koc, H. and Koc, E.C. (**June 2016**). Fyn-dependent phosphorylation of mitochondrial elongation factor Tu may lead to metabolic syndrome by mitochondrial dysfunction. NASA Research Day.

**Hunter, C.A.,** Koc, H. and Koc, E.C. **(March 2015).** c-Src regulates mitochondrial translation by elongation factor Tu (mtEF-Tu) phosphorylation. ASBMB/EB. Boston, MA. **ASBMB Travel Award.**

**Hunter, C.A.,** Koc, H. and Koc, E.C. (**March 2015**). **Oral Presentation.** c-Src regulates mitochondrial translation by elongation factor Tu (mtEF-Tu) phosphorylation. JCESOM Research Day. Huntington, WV.

**Hunter, C.A.,** Koc, H. and Koc, E.C. (**November 2014**). **Oral Presentation.** c-Src regulates mitochondrial translation by elongation factor Tu (mtEF-Tu) phosphorylation. ARCC. Huntington, WV.

**Hunter, C.A.,** Koc, H. and Koc, E.C. (October 2014). c-Src regulates mitochondrial translation by elongation factor Tu (mtEF-Tu) phosphorylation. Midwest Membrane Trafficking and Signaling Symposium (ASCB). Louisville, KY.

**Hunter, C.A.,** Miller-Lee, J.L, Koc, H and Koc, E.C. **(March 2014)**. Mitochondrial Elongation Factor Tu: Regulation Through Phosphorylation. JCESOM Research Day, Huntington, WV. **Basic Sciences Poster Presentation Award (1st)**.

**Hunter, C.A.,** Miller-Lee, J.L, Koc, H and Koc, E.C. **(October 2013)**. Mitochondrial Elongation Factor Tu: Regulation Through Phosphorylation. Appalachian Regional Cell Conference. Charleston, WV. **Poster Presentation Award (2nd)**.

**Hunter, C.A.** and C.A. Sarisky **(2011)**. Gene cloning in the purine biosynthetic pathway in *Archaeoglobus fulgidus.* Mid-Atlantic Regional Conference of Undergraduate Scholarships. Sweet Briar, VA.