**STAT3 Regulation of Citrate Synthase is Essential During the Initiation of Cell Growth**

By

Sarah MacPherson
B.Sc., University of Tulsa, 2013

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In the Department of Biochemistry and Microbiology

# Supervisory Committee

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**Outside Member**

# Abstract

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To exit a non-proliferative state and enter cell division, metazoan cells require external signals to facilitate activation and metabolic reprogramming. As cell growth is required before cell division, cells redirect their metabolism for *de novo* synthesis of cell building blocks, including phospholipids for cell membrane construction. How cells coordinate initial signaling events with metabolism is unknown. Lineage-specific factors transmit activating signals via cell surface receptor-ligand interactions. Among these are PI3K/AKT, MAPK/ERK, and JAK/STAT, all of which have been described to contribute to metabolic regulation. In particular, the signal transducer and activator of transcription (STAT) is a transcription factor with broad roles in cell cycle progression and glucose metabolism. Previous data from our laboratory found that one STAT family member, STAT3, was one of the primary signaling pathways activated when transitioning out of a resting state. Inhibition of STAT3 was found to suppress the initiation of cell growth and citrate levels, a main intermediate for fatty acid synthesis, suggesting a connection to cell metabolism. This thesis investigates the role of STAT3 in the regulation of metabolism in cells transitioning from a resting state to a cell growth state.

The first chapter of this thesis provides relevant background information on the metabolic and signaling pathways involved in a resting and cell growth state. It also provides data that supports an important role for STAT3 during initial cell growth. The

second chapter demonstrates the importance of STAT3 in multiple cell types using a small molecule inhibitor of STAT3, STAT3 knockdown, and knockout experiments. I also establish a potential link between STAT3 and the metabolic enzyme citrate synthase (CS) for the synthesis of citrate. In the third chapter I show that STAT3 transcriptionally regulates CS through two binding sites, CS1 and CS2. Finally, I determine that CS is essential for initial cell growth and that exogenous citrate can rescue the loss in cell growth and proliferation observed in the CS and STAT3 knockdown cells. Together, these findings describe a novel mechanism for initial cell growth whereby signaling and metabolic events are tightly linked to regulate the transition from a resting state to a state of initial cell growth. These results may uncover new strategies to block the initiation of proliferation in human pathological conditions including tumor recurrence and autoimmunity.

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# Acknowledgments

First, I would like to thank my supervisor Dr. Julian Lum. I am greatly appreciative of him taking me on as a graduate student as I had very little laboratory experience when I first entered the lab. Julian constantly challenged me throughout my Masters, allowing me to grow and adapt quickly into the research world. I also appreciate Julian's involvement as a supervisor, as he is always available to talk and will even help with experiments in laboratory. Julian does not only care about my development as a researcher, but as a person as well. He has been extremely supportive in my development as a researcher and of my dreams to become an Olympic athlete. His support and excitement about research made my experience in his lab extremely enjoyable. I have come a long way from my first timid months in my Masters and credit him for my development.

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# Chapter 1: Introduction

## 1.1 Prologue

This thesis aims to understand the signaling and metabolic pathways involved in initial cell growth. The introduction is designed to provide context to the research discoveries made in Chapters 2 and 3, and to highlight outstanding questions in the field of initial cell growth. First, I describe a principal signaling event that occurs when a cell transitions from a resting state to a cell growth state, and study the importance of this pathway. Next, I examine the metabolic pathway involved in initial cell growth and the importance of a specific metabolic enzyme, citrate synthase. Lastly I will determine a link between the primary signaling and metabolic events that occur during the initiation of cell growth.

In the introduction, Figures 3-6 provides preliminary evidence found by a previous member of the Lum Laboratory, Michael Horkoff. This preliminary work formed the basis of this thesis described in Chapters 2 and 3. Together, this data has been submitted as a manuscript for which I am the primary author.

## 1.2 Cell metabolism adapts to support cell homeostasis

Cell metabolism involves enzymatic reactions in which molecules are broken down to yield energy, or synthesized for maintenance of life. The balance between catabolism and anabolism can depend on the environmental pressures that a cell encounters, such as nutrient availability, intrinsic signaling factors, and extrinsic signaling factors (1–4). For example, a cell in nutrient and growth factor poor conditions will suppress anabolic pathways and promote catabolism, thereby inducing a resting state

(5,6). In contrast, growth factor stimulation under nutrient-rich conditions in mammalian cells results in upregulation of anabolic pathways that promote cell growth, proliferation and survival (4,7). This metabolic regulation is essential for cell homeostasis. An inability to respond to cellular signals and environmental pressures can lead to cell cycle arrest or apoptosis (8,9). Thus, it is important to understand what metabolic components are crucial to support changes in metabolism when responding to cellular demands.

### 1.2.1 Glycolysis supports the synthesis of key intermediates for bioenergetics and biosynthetics

Glucose is the main carbon source of the cell, providing intermediates for bioenergetics and biosynthetics. It is transported into the cell through glucose transporters, membrane proteins found on the surface of the cell (10). Once in the cell, glucose undergoes a series of enzymatic reactions in the cytosol and is converted to pyruvate independent of oxygen, termed glycolysis. This conversion first undergoes a consuming phase whereby adenosine triphosphate (ATP) is required, followed by a producing phase, resulting in the net production of two nicotinamide adenine dinucleotide hydrates (NADH) and 2 ATPs. The series of intermediates produced during glycolysis can be shunted to other metabolic pathways for nucleotide, amino acid and fatty acid synthesis. For example, glucose-6-phosphate can exit glycolysis and enter the pentose phosphate pathway supporting both fatty acid synthesis and nucleotide synthesis. Other intermediates of glycolysis, including 3-phosphoglycerate and phosphoenolpyruvate, are key precursors in the biosynthesis of amino acids (11). Pyruvate is the final product of glycolysis, which in the presence of oxygen will be converted to acetyl-CoA and continue

through the tricarboxylic acid (TCA) cycle to further support bioenergetics and biosynthetics of the cell.

### 1.2.2 The TCA cycle supports the synthesis of key intermediates for bioenergetics and biosynthetics

The preferential destination of glucose-derived carbons is the TCA cycle, located in the mitochondria of eukaryotic cells (12). Here, acetyl-CoA undergoes successive enzymatic reactions for the production of energy precursors. Besides GTP, the TCA cycle produces the protons and electron donors, NADH and $FADH_2$, which are utilized by the electron transport chain for oxidative phosphorylation and electron transport. The electron transport chain has the potential to synthesize up to 34 ATP from the TCA cycle, while only 4 ATP can be synthesized by glycolysis. The TCA cycle and the electron transport chain are therefore tightly linked as the TCA cycle supplies the majority of the protons and electrons utilized for ATP synthesis. Their relationship is vital, as neither are sufficient alone to accommodate the bioenergetic demands of a dividing cell (13).

Although the mitochondrion is the known energy hub of eukaryotic cells, it is also involved in the synthesis of intermediates for macromolecule production (12). For example, oxaloacetate and α-ketoglutarate (α-KG), two intermediates of the TCA cycle, are the main precursors for protein and nucleotide synthesis. In addition, citrate, the first intermediate of the TCA cycle after completion of glycolysis, is utilized for the synthesis of *de novo* fatty acids, an essential macromolecule for cell growth.

## 1.3 The transition from a resting state to a state of cell growth

A cell can be found in three main states: resting, growth or division. All three states require various levels and specificity of signals and metabolic events. The fate of each state requires that the metabolic requirements are met or the cell otherwise enters a less demanding state or commits cell death. These requirements are fairly well described for all three states. However, the requirements when transitioning between states is rather unclear, specifically when transitioning from a resting state to a state of cell growth.

### 1.3.1 The induction and regulation of a resting state

Resting cells (also referred to as quiescent or $G_o$ cells) differ from proliferative cells in that they do not divide but retain the ability to re-entre the cell cycle and proliferate (17). A resting state is found before cell differentiation, or can be induced under conditions that are unfavourable for proliferation (14). For example, some cell types like naïve T lymphocytes and hematopoietic stem cells are found in a resting state until stimulation through extrinsic signals (14). A resting state can also be triggered by nutrient limitation in bacteria and yeast (15). In mammalian cells, a resting state can be induced not only by nutrient limitation, but also by a host of other extracellular and intracellular factors (16). Although there are multiple avenues for the induction of a resting state, the key metabolic and signaling pathways utilized to maintain a resting state are similar.

Cell signaling and metabolic pathways are adjusted to support the demands of a cell once it enters into a resting state. Compared to logarithmic-phase yeast cultures, yeast entering a resting state decrease unnecessary transcriptional pathways (5). This

results in an overall transcription rate that is three to five times lower, reducing the synthesis of many proteins (5). Similar reductions in transcriptional rates are also observed in mammalian cells (17). The reduction of transcriptional pathways results in reduced expression of glucose transporters and cell metabolism (9). The reduction in glucose uptake results in decreased flux through glycolysis and the TCA cycle, reducing both bioenergetics and biosynthesics of the cell (6). The bioenergetics of a resting cell is supported by recycling intracellular components to promote survival, also known as catabolism (6,18–20). Thus, both signaling and metabolic pathways of a resting cell are found at basal levels in a resting state.

### 1.3.2 The induction of initial cell growth

Once environmental conditions are suitable to promote cell division, the cell will transition from a resting state to a state of cell growth. In yeast, exit from carbon withdrawal-induced quiescence relies on glucose catabolism and is independent of ATP production (21). This ensures that cells accumulate sufficient biomass or size before beginning DNA replication and cell division (15,22–25). Thus initial cell growth refers to the period between a stimulus to exit a resting state, and the first cell division.

Mammalian cells first require stimulation through cell surface receptors to exit a resting state. Signaling pathways specific to the stimulated receptor become activated and regulate the expression of key proteins to support the demands of cell growth. Unlike prokaryotes or yeast, mammalian cells depend on extrinsic factors for growth and proliferation. Resting T cells for example require stimulation through the T cell receptor with the appropriate antigen resulting in activation, proliferation and induction of an

immune response. Growth factors have been linked to both the regulation of cell metabolism and signal transduction (26,27). Signaling pathways specific to the stimulated receptor become activated and regulate the expression of key proteins to support demands of cell growth.

The transition from a resting state to a proliferative state requires that the cell first increase in size as cell division alone cannot increase total cell mass without cell growth. Cell size is an essential cellular element of all cell types, impacting cellular design, fitness and function. It is well established in a variety of eukaryotic cells that cell volume increases with ploidy, linking cell growth to cell cycle progression (28). To increase in size upon stimulation, cells will upregulate specific metabolic and signaling pathways to promote cell growth. As glucose is the main carbon source for mammalian cells, shortly after stimulation a significant increase in the glucose transporter GLUT1 is observed (29). This increased flux of glucose leads to increased glycolysis supporting initial cell growth (6). However, further research is required to determine what key metabolic and signaling pathways are important for the transition from a resting state to a state of cell growth.

### 1.3.3 Metabolic pathways that regulate cell growth

The shift from basal levels of metabolism in resting cells to an increase in glucose uptake and glycolysis seen in cells initiating proliferation leads to an increased supply of carbons for entry into the TCA cycle (Figure 1). Citrate is the first intermediate synthesized in the TCA cycle through the condensation reaction of oxaloacetate and acetyl coenzyme A (acetyl-CoA) by citrate synthase (CS) (Figure 1). Normally, citrate

undergoes successive enzymatic reactions in the TCA cycle to support cellular bioenergetics and biosynthetics. However in a state of initial cell growth, the cell will preferentially export glucose-derived citrate to the cytosol for *de novo* fatty acid synthesis for the production of lipids, supporting cell membrane construction (30–32). The dependency of citrate to fuel growth implies that in addition to maintaining high rates of glycolysis, specific steps of the TCA must be coordinately regulated to satisfy the demands for fatty acids. Thus, examination of the role of CS for the production of citrate during initial cell growth is required.

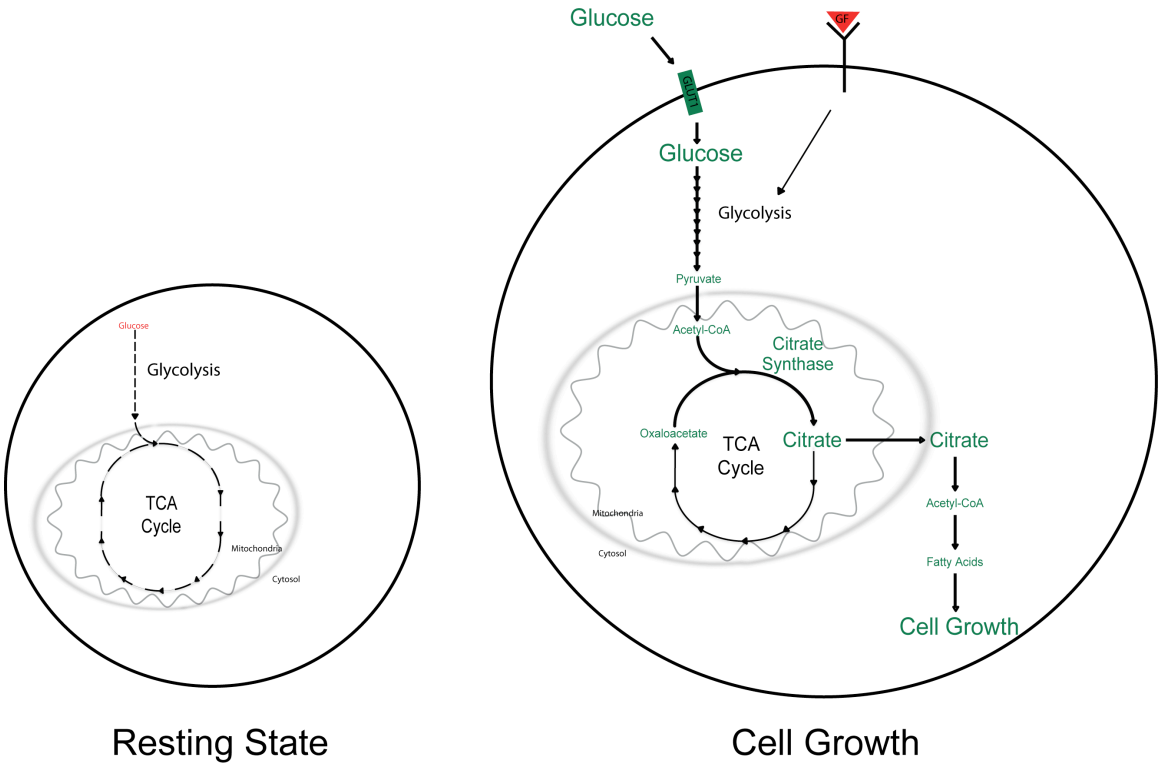


**Figure 1. Comparison of resting cell metabolism versus initial cell growth.** Resting cells decrease in cell size and maintain basal levels of energy through catabolic processes. Initial cell growth in mammalian cells can be stimulated upon growth factor binding (red triangle), leading to glucose uptake (green rectangle-GLUT1) and the synthesis of glucose derived citrate by CS in the mitochondria. Citrate is then exported to the cytosol for *de novo* synthesis of fatty acids, providing lipids for cell membrane construction.

### 1.3.3.1 Citrate synthase and its role in cell growth

Citrate synthase is a metabolic enzyme of the TCA cycle that is present in all-eukaryotic cells. CS catalyzes the reaction between acetyl-CoA, oxaloacetate and water to produce citrate and CoA without the direct participation of ATP or any other nucleoside triphosphates. In eukaryotes, mitochondrial CS is encoded by nuclear DNA, translated in the cytoplasm and then transported into the mitochondria where it is localized in the mitochondrial matrix. Citrate synthase is a single nuclear gene of 28,694 bases and in humans, is transcribed into two isoforms of 466 and 400 amino acids by alternative splicing of exon 2 (33). The more highly expressed larger isoform contains an amino terminal mitochondrial targeting sequence and can be found in the mitochondria (33). The structure of CS is conserved in animals, plants and fungi and consists of two identical subunits (34). In algae and gram-negative bacteria however, CS consists of four to six identical subunits (34).

The enzymatic reaction of CS involves a large conformational change during catalysis. The binding of oxaloacetate to CS results in a closed version of CS creating a binding site for acetyl-CoA. As metabolic flux is ultimately controlled by substrate availability, the levels of acetyl-CoA and oxaloacetate in the mitochondria controls the rate of the reaction. The standard free energy change for citrate synthase reaction is -31.5 kJ/mol suggesting that CS is likely to function far from equilibrium under physiological conditions. Therefore, CS is a rate-determining enzyme in the TCA cycle.

The regulation of CS occurs through multiple mechanisms (35). Besides regulation by the availability of acetyl-CoA and oxaloacetate, CS can be regulated by succinyl-CoA, ATP:ADP and NADH/NAD+ rations (35). The elevated levels of

ATP:ADP denote a high energy state of the cell, indicating that continuation in the TCA cycle through CS for energy production is unnecessary. Inhibition of CS by succinyl-CoA is due to its structural similarity with acetyl-CoA, allowing succinyl-CoA to act as a competitive inhibitor of the reaction.

Although CS is a nuclear transcribed gene, little is known about the transcriptional regulation of CS. In mammalian cells, transcriptional regulation of CS has been described to support myogenesis, as well as lipid metabolism in the liver (36,37). However, whether CS is transcriptionally regulated during the initiation of cell growth is yet to be described.

After the synthesis of citrate by CS, citrate diffuses from the mitochondria to the cytosol via the tricaboxylate carrier (SLC25A1) to promote fatty acid synthesis for cell growth. Citrate can support fatty acid synthesis through three pathways. First, increased levels of citrate, as well as ATP, can allosterically reduce the metabolic flux of glycolysis by inhibiting the glycolytic enzyme phosphofructokinase-1. This inhibition of glycolysis redirects metabolites to the pentose phosphate pathway, which supports the production of NADPH, an essential coenzyme for fatty acid synthesis. Secondly, once in the cytosol, citrate can be converted into acetyl-CoA and oxaloacetate by ATP citrate lyase. This is an essential initial event for the promotion of fatty acid synthesis, as acetyl-CoA is a main precursor for fatty acid synthesis. Lastly, citrate activates acetyl CoA carboxylase, an enzyme that controls fatty acid synthesis. Thus, citrate has the ability to organize metabolic pathways to promote fatty acid synthesis and cell growth.

CS has been described previously to be essential for cell growth and proliferation in some cell types. For example, the absence of CS in algae, plants, yeast, bacteria, and

worms has been observed to contribute to defects in meiosis and inhibition of growth (38–42). Some evidence also supports a potential role for CS in initial cell growth and differentiation. For example, in bacteria and plants, citrate has been described to be produced during the first growth phase (43) and that loss of CS caused growth defects and decreased flowering in plants (44). In addition, an increase in CS activity has been observed in hematopoietic cells after acute mitogen stimulation (45). Despite what has been reported on the role of CS in growth and proliferation, much less is known about the role of CS during the earliest periods when resting cells begin to resume cell growth.

## 1.4 Growth factors and metabolism

Growth factors are required to sustain glucose metabolism and promote cell survival, as reductions in growth factors leads to decreases in glucose transport, cell size and glycolysis (4). Growth factor signal transduction impacts glucose utilization in multiple ways. First, glucose uptake by the glucose transporters Glut1 and Glut4, have been shown to be controlled by growth factors (46,47). Growth factor regulation of glycolytic enzymes including hexokinase and phosphofructokinase, have been shown to effect the glycolytic rate (4). These increases in glucose transport and glycolytic rate supports cell growth and proliferation. The initial metabolic events following initial stimulation with growth factors have been previously studied. Rat lymphocytes stimulated with lectins and interleukins have a 53-fold increase in glucose metabolism (48,49), while the activity of individual glycolytic enzymes increased 12- to 30 fold upon stimulation (50). These data support the importance of glucose and glycolysis following growth factor stimulation.

## 1.5 Growth factors and signaling

Proliferation in normal mammalian cells is regulated not only by the presence of nutrients but also by cues from proliferative signaling molecules like mitogens or growth factors that interact with receptors localized at the plasma membrane. There are many classes of cell surface receptors used to accommodate the extrinsic signals found in the cellular environment. For instance, receptor tyrosine kinases, one of six known members of enzyme-linked receptors, has 90 unique genes identified in the human genome and displays high affinity for a range of polypeptides, cytokines and growth factors (51). These receptors have kinase activity leading to signal transduction and transcriptional regulation of various genes. Growth factors have specificity for certain receptors, signal transduction pathways and thus gene targets. Although there are many families of growth factors, here I focus on IL-2 and IL-3.

Interleukin 2 (IL-2) is a T cell growth factor that is well known to promote cell differentiation. The IL-2 cytokine binds to the IL-2 receptor, which is comprised of an alpha, beta and gamma chain. While all three IL-2 receptor chains extend into the cell, only the beta and gamma chains participate in signaling with the tyrosine kinase family JAK. JAK has been described to be involved in the activation of the intracellular signaling pathways PI3K/AKT, MAP/ERK, and JAK/STAT (52,53).

Alternatively, IL-3 can stimulate proliferation of all non-lymphoid cells. Similar to IL-2, IL-3 cytokines bind to the IL-3 receptor that is comprised of an alpha subunit that is ligand specific, and a beta subunit for signal transduction. When IL-3 binds to its receptor, adapter proteins are recruited for the activation of signal transduction pathways. Activation of the MAP/ERK pathway by IL-3 is known to promote cell growth and

differentiation (54), whereas activation of the PI3K/AKT pathway by IL-3 has been described to suppress apoptosis (55). IL-3 stimulation has also been linked to JAK2 activation, which in turn phosphorylates STAT5 for the transcription of genes involved in cell differentiation and survival (56,57).

## 1.6 Signal transduction pathways that control metabolism

The signaling and metabolic pathways that govern proliferating metazoan cells have been well studied. PI3K/AKT, MAP/ERK, and JAK/STAT pathways are a few key pathways that are studied in this thesis and that are known to contribute to metabolic regulation of carbon sources for the synthesis of essential cell macromolecules like nucleotides, proteins and lipids (27). However, the signal transduction pathway that is essential for initial cell growth following growth factor stimulation is unknown.

### 1.6.1 PI3K/AKT/mTOR

The PI3K/AKT/mTOR intracellular signaling pathway is important for regulating cellular quiescence, growth, differentiation, survival and proliferation in plants and mammalian cells (58–60). Phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3K) are a family of intracellular signal transducer enzymes found in the cytosol. Growth factor binding and receptor tyrosine autophosphorylation provides a docking site for PI3K activation. Activation of PI3K can then lead to phosphorylation of AKT (protein kinase B), a major downstream effector of PI3K, leading to multiple downstream signaling events that regulate many cell events, including the cell cycle (61) (Figure 2).

AKT is a serine/threonine-specific protein kinase known to influence cell survival and metabolism through regulation of downstream effectors. AKT has been shown to post-transcriptionally support multiple steps in glycolysis (62). For example, PI3K/AKT promotes trafficking of glucose transporters to the cell surface and increases the activity of several glycolytic enzymes (62–64). The activation of AKT in tumor cells has been shown to overcome cell cycle arrest in G1 phase (65). While in T cells, overexpression of AKT has been shown to promote resting T cell growth and proliferation (66).

mTOR (mammalian target of rapamycin) is a serine/threonine protein kinase conserved in all eukaryotes that integrates extrinsic and intrinsic signals related to nutrient levels, energy status, and stress to induce changes in cellular metabolism, growth, and proliferation (67). mTOR promotes anabolic processes, creating *de novo* cell building blocks like proteins, nucleic acids and lipids, while inhibiting catabolic processes like autophagy. mTOR has been described to be critical for determining the metabolic state of mammalian cells (68,69). Thus the PI3K/AKT/mTOR pathway appears as though it could be a key signaling pathway involved in the initiation of cell growth and proliferation.

### 1.6.2 MAPK/ERK

The MAPK/ERK signaling cascade is a known regulator of the cell cycle (70). The pathway is activated through a wide variety of receptors involved in cell growth and differentiation, including receptor tyrosine kinases and the T cell receptor. A wide variety of extrinsic factors are involved in ERK activation, including the cytokine IL-3 (71). An adaptor (Grb2) links the receptor to a guanine nucleotide exchange factor (Sos),

transducing the signal to small GTP binding proteins (Ras). This then leads to the activation of the kinase cascade ending in phosphorylation of the protein kinase signaling molecule MAPK (mitogen-activated protein kinases) or ERK (extracellular signal-regulated kinases) (Figure 2). Phosphorylation at both Thr202/Tyr204 sites is required for full ERK activity. Once activated, ERK will translocate to the nucleus for regulation of its target genes.

ERK has been described to regulate cell growth by promoting the activity of cell cycle regulators including D-type cyclins, while suppressing other cell cycle regulators such as p21, p27 and p15 (72). ERK has also been suggested to regulate metabolism through pyruvate dehydrogenase and through the regulation of glutamine uptake and nucleotide synthesis (73,74). The key regulatory role for MAPK/ERK in metabolism and cell cycle control suggests that it could be involved in the initiation of cell growth.

### 1.6.3 JAK/STAT

The JAK/STAT signaling pathway is involved in growth factor and gp130-mediated cytokine signaling, both of which are required for activation of key cellular processes such as the cell cycle, survival and proliferation (75). There are 7 STAT family members (STAT1-STAT6). STAT3 and STAT5 are expressed in most cell types and are activated by a variety of growth factors, while other STAT proteins play specific roles in host defense. STAT5 has been described to be activated by JAK1 or JAK3 following IL-3 stimulation promoting cell growth, division, apoptosis and cell differentiation (57,76). STAT3 is also known to have an essential role in many pro-survival pathways and in early embryogenesis, as STAT3 deficient mice die prior to gastrulation (77).

The STAT3 pathway can be activated by multiple growth factors, although the role of IL-6 for STAT3 activation has been highly investigated(78). After growth factor stimulation, STAT3 is phosphorylated by JAK2 through binding to the gp130 ligand binding subunit on the SH2 domain for tyrosine phosphorylation. Once phosphorylated, cytosolic STAT3 will hetero or homodimerize to another phosphorylated STAT, leading to its translocation to the nucleus for transcriptional regulation of its target genes (Figure 2). STAT3's target genes are involved in many cell survival pathways including anti-apoptosis (Bcl-2, p53, Bcl-$X_L$), cell division (Cylin D1, Myc), angiogenesis (VEGF, HIF-1a) and inflammation (IL-6, IL-11, IL-17, CXCL12)(79,80).

The STAT3 signaling pathway was first observed to be involved in cellular respiration when STAT3 deficient pro-B cells where found to have defects in complex I and II of the electron transport chain (81). STAT3 has been described to transcriptionally regulate metabolic factors including the glucose transporter GLUT1 and the transcription factor HIF-1a, both of which promote glycolysis (82). Thus the JAK/STAT pathway may also have an important role in the initiation of cell growth and proliferation.

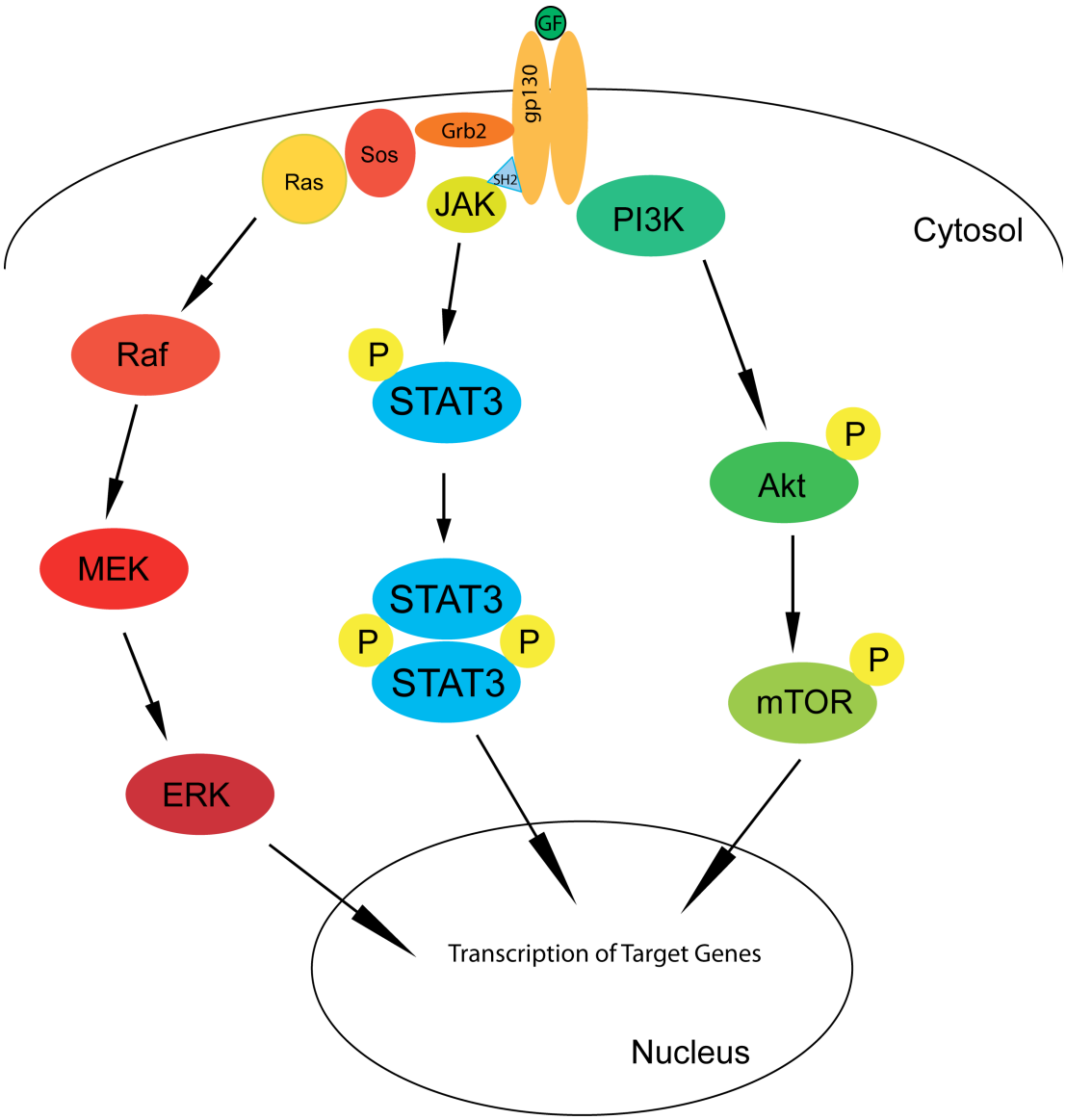


**Figure 2. Known signaling pathways involved in cell growth and proliferation.** The MAP/ERK, JAK/STAT and PI3K/AKT/mTOR signaling pathways are all associated with cell growth and proliferation through different transcriptional target genes. All pathways become activated first by specific growth factor binding followed by tyrosine kinase activity. The further downstream signaling events are simplified above, as there are various routes of cross activation into other pathways.

## 1.7 Investigating the mechanism of initial cell growth

To study the transition from a resting state to a cell growth state requires a system whereby the cells are capable of entering a non-proliferative state indicative of decreased

signaling, metabolism and cell size. These resting cells must also retain the ability to initiate proliferation upon stimulation. As stated previously, limiting nutrients or growth factors can induce a resting state, however these methods often lead to a loss in cell viability (4). This results in poor environmental conditions for cells attempting to enter a resting state and limits the number of cells available to study the transition (83). Thus, I used two different systems to study the transition between a resting state and initial cell growth. In the first, I stimulated naïve T cells through the T cell receptor and examined cell growth and proliferation. In the second I induced a resting state by withdrawing growth factors from an apoptosis deficient, IL-3 dependent cell line, followed by the reintroduction of IL-3 to initiate proliferation.

### 1.7.1 Stimulation of splenocytes

Naïve T cells are found in a resting state, with basal levels of metabolism and signals, awaiting stimulation through T cell receptor with the appropriate antigen. Once activated T cells become highly proliferative, creating an immune response against the antigen presented. Studies show that naïve T cells stimulated in culture induces rapid division occurring one day post stimulation and can continue through multiple rounds of division for up to four days (84). T cell proliferation *in vitro* can be induced by T cell receptor stimulation along with IL-2, a well known potent T cell growth factor (85). This stimulation will promote signal transduction pathways to promote naïve T cell differentiation, survival and proliferation (86). Therefore, I used a model whereby naïve CD8+ or CD4+ T cells were isolated from the spleen of a mouse and were stimulated *in*

*vitro* with anti-CD3/anti-CD28 and IL-2, which mimics stimulation by antigen presenting cells. This allows for the study of initial cell growth.

### 1.7.2 An apoptosis deficient, IL-3 dependent cell model

The second model utilizes an IL-3 dependent hematopoietic cell line isolated from the bone marrow of apoptosis deficient mice $Bax^{-/-}$ $Bak^{-/-}$(DKO). Mice that lack both Bax and Bak die prenatally with fewer than 10% surviving into adulthood (87). Phenotypes of these apoptosis deficient mice include interdigital webs, excess cells in the central nervous and hematopoietic systems, deafness, circling behaviour, increased number of lymphocytes, and massive spleens and lymph nodes (87). Cells isolated from the bone marrow of these mice were immortalized and cultured in IL-3, resulting in an IL-3 dependent hematopoietic cell line.

As stated previously, removal of growth factors leads to apoptosis in mammalian cells. However, the DKO cell line described above lacks two apoptosis genes and thus cannot commit apoptosis. DKO cells have been shown to survive several weeks under IL-3 withdrawal (6). It has also been shown that DKO cells under IL-3 withdrawal become dependent on catabolic processes and decrease in cell size without loss in cell number or viability (6). Upon IL-3 readdition, all cells restore their glycolytic capacity and are able to resume cell growth and proliferation (6). This model permitted a closer examination of the sequence of signaling events that regulate cell growth during the initial period in response to IL-3 stimulation. Early studies in our lab examined the signaling events that coordinate initiation of cell growth and proliferation.

## 1.8 Previous Findings

Our current knowledge of the signaling and metabolic pathways involved in initial cell growth is limited. Although, it is understood that following growth factor stimulation a cell must increase in size in order to divide. I therefore believe that there is a link between the initial signal transduction pathways and the metabolic events that govern cell growth. To determine the mechanism by which cells initiate cell growth, our lab first determined the signaling pathway that is important following growth factor stimulation, and whether this signaling pathway influences a specific metabolic event involved in initial cell growth.

### 1.8.1 STAT3 is the primary signaling pathway induced following growth factor readdition

To investigate the expression of several key regulators of cell growth, AKT, ERK and STAT3 were examined in DKO cells by immunoblotting in the absence of IL-3 and at various time points post IL-3 readdition (78,88,89). These three major signaling cascades are activated by IL-3 and are known to regulate cell growth through both direct and indirect mechanisms (90,91). Both AKT and ERK were suppressed in DKO cells that were cultured in the absence of IL-3 for 21 days, with the exception of STAT3 (Figure 3). This preserved expression of STAT3 during IL-3 withdrawal is consistent with previous findings (6). Upon IL-3 readdition, STAT3 phosphorylation was detected within the first hour (Figure 3)(148). In contrast, the phosphorylation of AKT and ERK1/2 did not occur until 72 hours after IL-3 readdition, a time point at which cells have increased in size (6).

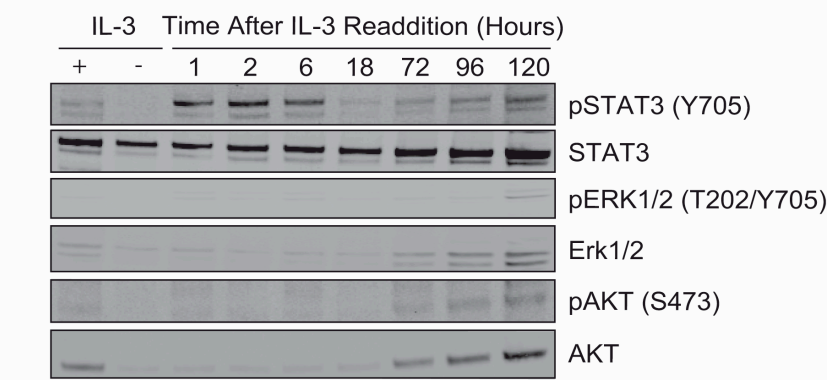


**Figure 3. STAT3 is the primary signaling pathway induced following growth factor readdition.** Immunoblot of DKO cells in the presence of IL-3 (+), in the absence of IL-3 for 21 days (-), and hours after IL-3 readdition. The phosphorylated form of STAT3 (pSTAT3), as well as total STAT3 was analyzed(148).

### 1.8.2 Chemical inhibition of STAT3 suppresses initial cell growth and proliferation

To study the importance of early STAT3 activation on cell growth, a small molecule chemical inhibitor of STAT3, WP1066 was used (92). WP1066 is a tyrosine kinase inhibitor with specificity for STAT3 at the micromolar range, blocking the phosphorylation and activation of the STAT3 pathway (92). To study the effects of WP1066 on initial cell growth and proliferation, DKO cells were starved of IL-3 for 14 days and were treated with WP1066 at the time of IL-3 readdition. The activation of STAT3 as assessed by tyrosine 705 phosphorylation following IL-3 stimulation was suppressed by treatment with WP1066, while phosphorylation and total STAT5 levels remained largely unaffected 2 days after IL-3 readdition (Figure 4A)(148). Treatment with WP1066 also blocked the recovery of cell size and the capacity to proliferate in response to IL-3 (Figures 4B and 4C)(148).

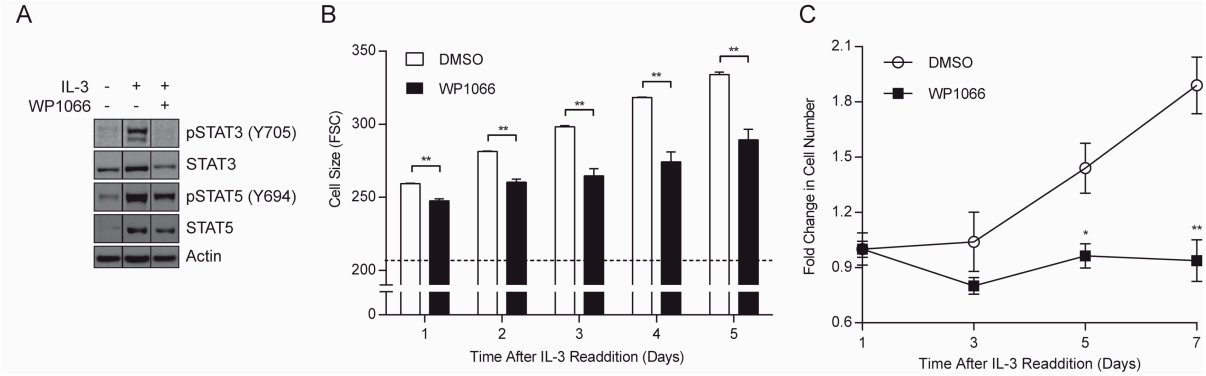


**Figure 4. STAT3 inhibitor supresses initial increases in cell size and proliferation.** (A) Immunoblot of phosphorylated and total STAT3 and STAT5 in the absence of IL-3 for 14 days (-) and one day after IL-3 readdition (+) in the absence (-) or presence (+) of WP1066. (B) Cell size (FSC units) in DKO cells after IL-3 readdition in the presence (black bar) or absence (white bar) of WP1066. (C) Fold change in cell number after IL-3 readdition in the presence (black square) or absence (white circle) of WP1066. Fold change was calculated by dividing the IL-3 readdition cell number by the 14 days IL-3 deprived cell number. Graph shows average ± SEM (n=3, Student's *t*-test, *p < 0.05, **p < 0.01)(148).

### 1.8.3 Chemical inhibition of STAT3 supresses mitochondrial membrane potential which can be rescued by the addition of exogenous α-ketoglutarate

To determine the effects of STAT3 inhibition on mitochondrial function, flow cytometric analysis of mitochondrial repolarization was measured by tetramethylrhodamine ethyl ester (TMRE). TMRE is a lipophilic cation dye that accumulates in the intermembrane space proportionally to the cell membrane potential. IL-3 deprived cells treated with WP1066 had a significant impairment in their recovery of mitochondrial potential after IL-3 readdition (Figure 5A)(148). This was not due to a loss in mitochondrial mass, as there was no difference in Mitotracker staining between the WP1066 treated versus control cells (Figure 5B)(148).

Recent studies suggest that mitochondrial STAT3 has a direct regulatory role in oxidative metabolism and electron transport due to the decrease in complex I and II activity observed in $STAT3^{-/-}$ cells (81,93–95). Alternatively, STAT3 may control other upstream metabolic events that modify the flux of metabolites (*e.g.* pyruvate) or energy equivalents (*e.g.* NADH, $FADH_2$) used to maintain mitochondrial membrane polarization or synthesis of *de novo* fatty acids. To distinguish between these possibilities, DKO cells were deprived of IL-3 for 14 days. At the time of IL-3 readdition, the cells were treated with WP1066 and 2 days later the cells were cultured in the presence or absence of the cell-permeable metabolites methyl-pyruvate (MP) or dimethyl-2-oxoglutarate (MOG). Six hours after incubation with MP or MOG, the mitochondrial membrane potential was measured by TMRE (Figure 5C)(148). We expected that MOG, an α-ketoglutarate analog, would serve as an anapleurotic substrate and rescue the loss in membrane potential by generating NADH through successive decarboxylation reactions to produce oxaloacetate (Figure 5D)(148). As shown above, WP1066 alone prevented the recovery of mitochondrial membrane potential two days after IL-3 readdition, while the addition of MP led to no significant TMRE recovery. However, in cells treated with WP1066, the addition of MOG was able to rescue the loss in mitochondrial potential (Figure 5E)(148).

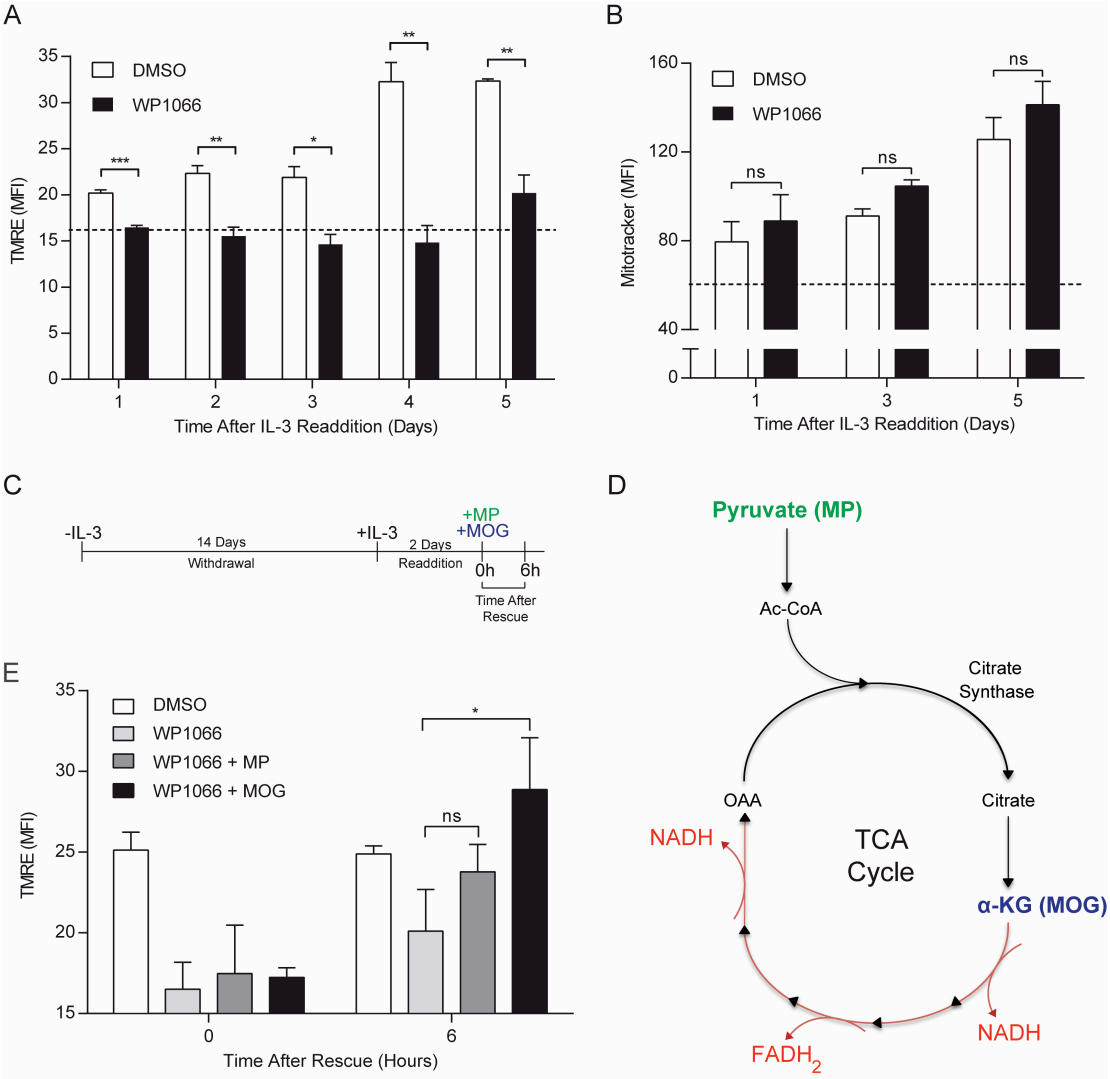


**Figure 5. Loss of mitochondrial membrane potential by STAT3 inhibition is rescued by α-ketoglutarate.** DKO cells were cultured in the absence of IL-3 for 14 days (A) mitochondrial membrane potential measured by TMRE after IL-3 readdition in the presence (black bar) of absence (white bar) of WP1066. (B) Mitochondrial mass days after IL-3 readdition in the presence (black bar) or absence (white bar) of WP1066. (C) Schematic of IL-3 withdrawal and readdition for metabolic substrate rescue of TMRE. Pyruvate (MP) or α-ketogluratate (MOG) was supplemented into the media 2 days after IL-3 readdition followed by TMRE staining. Six hours post-MP or –MOG addition, cells were collected for TMRE analysis by flow cytometry. (D) Metabolic model for α-ketoglutarate and pyruvate rescue in mitochondrial TMRE. (E) Mitochondrial membrane potential measured by TMRE two days after IL-3 readdition in the presence of WP1066 alone (light grey bar), with the addition of pyruvate (MP) for 6 hours (dark grey bar), with the addition of α-ketogluratate (MOG) for 6 hours (black bar), or DMSO control (white bar); representative of 3 independent experiments. Graph shows average ± SEM (n=3, Student's *t*-test, ns-not significant)(148).

### 1.8.4 Chemical inhibition of STAT3 supresses initial increases in citrate synthase expression and intracellular citrate levels

The ability of MOG, but not MP, to rescue the mitochondrial membrane potential led to the speculation that STAT3 controls the level of intracellular citrate. To examine this possibility, the level of intracellular citrate was measured 48 hours after IL-3 readdition in the presence or absence of WP1066. At this time point, citrate had recovered to levels similar to cells cultured in the continued presence of IL-3 (Figure 6A)(148). In contrast, the inhibition of STAT3 with WP1066 led to a dramatic loss in the ability to accumulate citrate over the same time course. The reduction in total citrate levels suggested that the expression of STAT3 regulates the production citrate. Indeed, CS mRNA levels increased 5-fold following IL-3 readdition (Figure 6B)(148). However, the upregulation in CS mRNA was blocked in DKO cells treated with WP1066. Consistent with this, immunoblotting revealed that between 24 and 48 hours after IL-3 readdition, the amount of CS protein increased but this was suppressed when DKO cells were cultured in the presence of WP1066 (Figure 6C)(148).

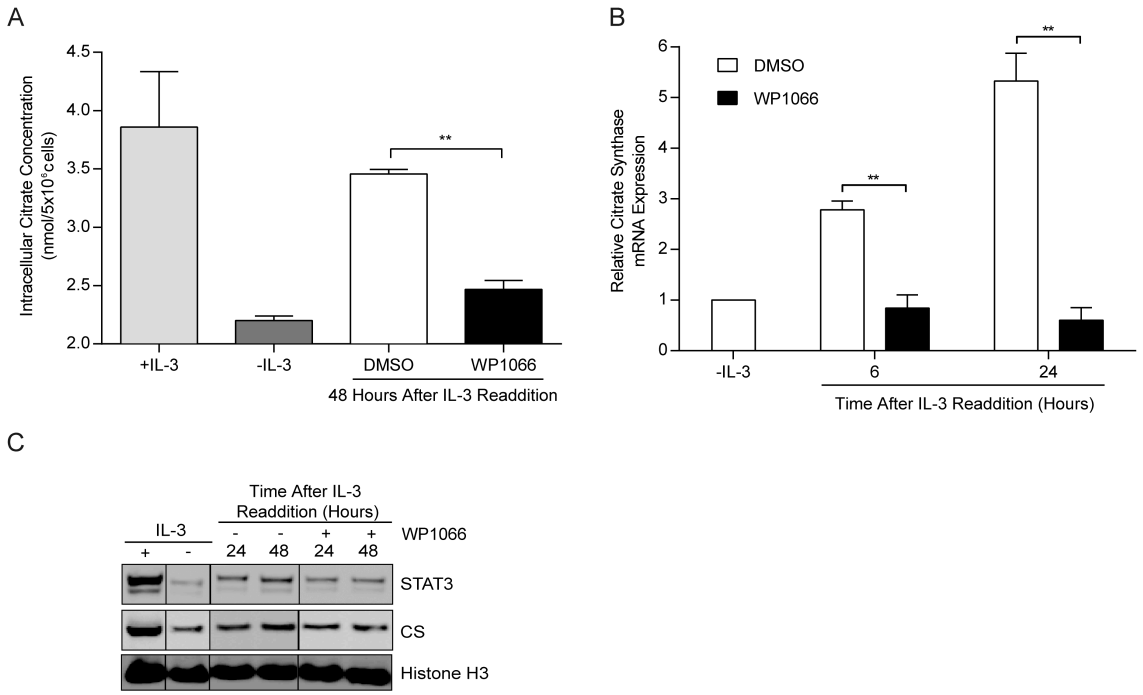


**Figure 6. Inhibition of STAT3 leads to loss in citrate and CS levels.** DKO cells were cultured in the absence of IL-3 for 14 days (A) Intracellular citrate levels in the presence of IL-3 (light grey bar), 14 days post IL-3 withdrawal (dark grey bar), and 48 hours after IL-3 readdition in the absence (white bar) or presence (black bar) of WP1066. (B) Relative CS mRNA expression measured by qPCR 14 days post IL-3 withdrawal (-IL-3) and 6 and 24 hours after IL-3 readdition in the absence (white bar) or presence (black bar) of WP1066. (C) Immunoblot shows total CS and STAT3 protein levels in the presence of IL-3 (+IL-3), 14 days post IL-3 withdrawal (-IL-3), and 24 and 48 hours after IL-3 readdition in the absence (-) or presence (+) of WP1066, representative of 3 independent experiments. Graph shows average ± SEM (n=3, Student's *t*-test, **p < 0.01)(148).

## 1.8.5 Conclusions from previous findings

The experiments described above provide the knowledge that most signaling pathways with the exception of STAT3 are suppressed during IL-3 withdrawal and that upon readdition, STAT3 is one of the first signaling pathways to be activated. Further experiments with the small molecule STAT3 chemical inhibitor WP1066 support the essential role for STAT3 during initial cell growth, as the inhibition of STAT3 leads to an

inability to initiate cell growth and proliferation. Further analysis into the role of STAT3 on cellular metabolism during the initial stages of growth found that citrate, CS mRNA and CS protein levels were suppressed. Overall this suggests that STAT3 could have either a direct or indirect regulatory role on CS in order to promote the synthesis of citrate, support fatty acid synthesis, initial cell growth and proliferation. Thus, Chapter 2 and 3 will further investigate the importance of STAT3 and CS on initial cell growth and determine whether STAT3 regulates CS during initial cell growth.

## 1.9 Summary and hypothesis

In order to exit a non-proliferative state metazoan cells require external signals, instructing activation and metabolic reprogramming to meet the demands of cell division (27). This reorganization of metabolism is necessary for initiation of cell growth and requires the *de novo* synthesis of cell building blocks including phospholipids for cell membrane construction through glucose derived citrate by CS (96). How cells coordinate initial signaling events with metabolism is unknown. Lineage-specific factors transmit activating signals via cell surface receptor-ligand interactions. Among these are PI3K/AKT, MAP/ERK and JAK/STAT all of which have been described to contribute to metabolic regulation (27,82). In particular, STAT3 is a transcription factor with broad roles in cell activation and glucose metabolism. Specifically, STAT3 has recently been shown to localize to the mitochondria and regulate the electron transport and cellular bioenergetics through an unknown mechanism (81,82,94,95).

Previous findings in our lab show that STAT3 was one of the first signaling pathways to be induced before any detectable changes in cell size were observed. Pharmacological inhibition of STAT3 at the time of IL-3 readdition resulted in an inability to initiate cell growth and proliferation. Notably, pharmacological inhibition of STAT3 also prevented the up-regulation of citrate synthase (CS) expression, a key enzyme required for the generation of citrate, the main carbon precursor for fatty acid biosynthesis (30).

These data suggested that STAT3 may control some critical aspect of metabolism during the earliest phases of cell growth. I rationalized that STAT3 might regulate the generation of precursors or the metabolic enzymes required for *de novo* fatty acid synthesis. In my thesis, I hypothesize that STAT3 regulates initial cell growth by controlling the expression of CS. I first aim to determine the effect of STAT3 signaling on cell growth and CS expression. I next aim to determine the mechanism of CS regulation by STAT3 and the importance of CS expression during the initiation of cell growth. These studies may uncover new strategies to block the initiation of proliferation in human pathological conditions including tumor recurrence and autoimmunity.

# Chapter 2: STAT3 is Essential for Initial Cell Growth and Proliferation

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J.J.L. and S.M. conceptualized and conducted the project. J.J.L. and S.M. contributed 5% and 95% respectively for the performed experiments. J.J.L., S.M., M.H., T.H. and J.Z. contributed 45%, 40%, 5%, 5%, 5% respectively in experimental design and scientific suggestions. All authors edited the manuscript.

## 2.1 Abstract

Cell signaling is a crucial component to cell growth and differentiation. However, the signaling events that occur during initial cell growth are poorly described. Previous data demonstrates that STAT3 is amongst the first signaling events to be activated in resting hematopoietic cells following growth factor stimulation, and that inhibition of STAT3 leads to reduced cell growth and proliferation. Here, we further show that STAT3 is essential for initial cell growth and proliferation using small molecule inhibition, shRNA knockdown and genetic ablation in a model system. Furthermore the importance of STAT3 in this process was not cell line-specific, as STAT3 inhibition attenuated cell growth and proliferation in hematopoietic cells, murine CD4+ and CD8+ T cells, as well as in human peripheral blood mononuclear cells. Interestingly, as a consequence of STAT3 loss, the enzyme citrate synthase as well as its synthesized product citrate were consistently found at suppressed levels. These results support an essential role for STAT3 during the initiation of cell growth and may provide a link between the metabolic and signaling events that occur during this process.

## 2.2 Introduction

Signal transducer and activator of transcription, STAT3, is a well-known member of the JAK/STAT signaling pathway. Through the stimulation of a diverse array of growth factors and cytokines, STAT3 promotes many cell survival pathways (75). Targeted disruption of the STAT3 gene has been shown to be embryonically lethal in mice (77). Moreover, loss of function mutations in the STAT3 gene leads to suppressed immunity, such as that observed with Hyperimmunoglobulin E Syndrome, a hereditary condition that is associated with recurrent lung, sinus, and skin infections (97). On the contrary, gain of function mutations in STAT3 can lead to the onset of autoimmune diseases (98). The induction of these conditions as a consequence of STAT3 alterations supports the importance of STAT3 in development and immune cell differentiation.

Although there is a significant understanding of the signaling events that occur during the cell's resting and growth states, very little is known about the pathways involved in the transition between these two phases. Initial cell growth denotes the period between a stimulus and the first cell division. Previous data in our laboratory using an apoptosis deficient, IL-3 dependent cell line (DKO) found that STAT3 was activated within the first hour of IL-3 stimulation, before any changes in cell growth (Figure 3). This suggested that STAT3 might be involved in the regulation of initial cell growth. Supporting this, inhibition of STAT3 was shown to supress initial cell growth and proliferation (Figure 4B and 4C). One potential explanation for the developmental importance of STAT3 is through the regulation of metabolism. STAT3 has been previously found to associate with complex I and II of the electron transport chain to mediate metabolic reprogramming of Ras-induced transformation of mouse embryonic

fibroblasts (81,94,95). However, whether STAT3 regulates metabolic pathways associated with fatty acid synthesis to support initial cell growth is currently unknown.

Recent work has been aimed at investigating the link between various signaling and metabolic pathways (27); however, the signaling pathways that regulate early metabolic events during the initiation of cell growth are still unknown. Cell division requires the synthesis of basic cell building blocks such as amino acids, nucleic acids and lipids. However before cell division can occur, the cell must first increase in size, via fatty acid synthesis for cell membrane construction. The sole carbon source for *de novo* fatty acid synthesis is through glucose-derived citrate. Citrate is an intermediate of the tricarboxylic acid cycle that is synthesized through a condensation reaction between oxaloacetate and acetyl-CoA, a process that is catalyzed by the enzyme citrate synthase (CS). Interestingly, pharmacological inhibition of STAT3 prevents the up-regulation of CS mRNA and protein expression (Figure 6A and 6B). Citrate levels were also found to be decreased in cells treated with a STAT3 inhibitor (Figure 6C). Therefore, we hypothesized that reduced citrate levels lead to the inability of the cell to increase in size and subsequently proliferate in response to growth factor stimuli when STAT3 is suppressed. Taken together, these findings imply that STAT3 may play an important role in the modulation of growth and proliferation through the regulation of cell metabolism.

Here, I further demonstrate the effect of STAT3 inhibition on initial cell growth in murine CD8+ T cells and human peripheral blood mononuclear cells (PBMCs). Additional experiments were performed to determine the effect of shRNA knockdown of STAT3 on initial cell growth, proliferation, CS expression and citrate levels. The results

of these initial studies were also confirmed following genetic ablation of STAT3 in murine CD4+ T cells.

## 2.3 Methods

*2.3.1 Cell culture and T cell isolation*

Unless otherwise indicated, the IL-3-dependent $Bax^{-/-}$ $Bak^{-/-}$ hematopoietic cell line (DKO) was used. DKO cells were maintained in complete media containing RPMI 1640 supplemented with 10 % FBS (Hyclone), 100 U/mL Penicillin/Streptomycin (Hyclone), 1 mM HEPES (Hyclone), 2 mM L-glutamine (Hyclone) and 3.5 ng/mL recombinant mouse IL-3 (BD Pharmingen). For growth factor starvation experiments, cells were washed and cultured for the indicated time in complete media in the absence of IL-3. At the time of IL-3 readdition, DKO cells were centrifuged and the media was replaced with fresh complete media containing IL-3.

CD8+ T cells were purified from the spleens of C57BL/6 mice by negative selection using MACs column (Miltenyi Biotec) or EasySep (Stemcell Technologies) magnetic bead separation. Enriched CD8+ T cells were cultured in RPMI 1640 media containing 10 % FBS (Hyclone), 100 U/mL Penicillin/Streptomycin (Hyclone), 1 mM HEPES (Hyclone), 2 mM L-glutamine (Hyclone) and 100 IU IL-2 (eBioscience). Cells were plated at 500,000 cells per well in a 96 well plate containing plate-bound anti-CD3 (1 ug/mL, clone OKT3) and anti-CD28 (0.5 ug/mL, clone RUO) (BD Pharmingen).

To overcome the requirement of STAT3 during embryogenesis, *CD4-Cre-*$STAT3^{fl/fl}$ mice were created by conditional knockout using the loxP-Cre recombinase system (99). *CD4*+ T cells were purified from freshly harvested spleens of *CD4-Cre-*

*STAT3*$^{fl/fl}$ conditional knockout mice by negative selection using EasySep magnetic bead separation (Stemcell Technologies) and cultured as above. Spleens were generously supplied by Drs. John O'Shea and Yuka Kanno.

Human peripheral blood mononuclear cells (PBMCs) were cultured in RMPI 1640 media containing 10% FBS (Hyclone), 100 U/mL Penicillin/Streptomycin (Hyclone), 1 mM HEPES (Hyclone), 2 mM L-glutamine (Hyclone), 1μg/mL PHA (Sigma-Aldrich) and 50 IU recombinant human IL-2 (eBioscience). Cells were plated at 250,000 cells per well in a 96 well plate.

### *2.3.2 Reagents*

Citrate assays were conducted according to the manufacturer's protocol (Abcam). Trypan blue exclusion assay (Sigma) or 123 count eBeads (eBioscience) were used to determine cell numbers. Methyl-pyruvate (MP) (5 mM), dimethyl-2-oxoglutarate (MOG) (5 mM) and sodium citrate (10 mM) were purchased from Sigma. WP1066 (10 μM, 8 μM or 0.5 μM) was purchased from Calbiochem.

### *2.3.3 Flow cytometry*

To measure mitochondrial membrane potential, cells were incubated with 10 nM tetramethylrhodamine, ethyl ester (TMRE) for 20 minutes at 37 °C prior to flow cytometric analysis. Cell size was determined by forward scatter (FSC). All data were collected on a BD FACSCalibur$^{TM}$ flow cytometer (BD Bioscience) and analyzed using FlowJo version 10 Software (Tree Star Inc.).

*2.3.4 Immunoblotting*

Cells were lysed with RIPA buffer containing 1X cOmplete™ EDTA-free protease inhibitor (Roche) and cocktail I and II phosphatase inhibitors (Thermo Scientific). Alternatively, cells were boiled in SDS lysis buffer containing protease inhibitors. Lysates were mixed with 10X NuPAGE Sample Reducing Agent and 4X NuPAGE LDS Sample Buffer (Invitrogen), boiled at 70 °C for 10 minutes and resolved on a pre-cast NuPAGE 4-12 % BisTris gel (Invitrogen) in 1X MES SDS Running Buffer. Proteins were transferred onto nitrocellulose membrane (Life Sciences) and blocked in 5% skim milk in TBS. Immunoblots were probed with the following antibodies: pSTAT3 (Y705), STAT3, pSTAT5 (Y694), STAT5, pAKT (S473), AKT, pERK1/2 (T202/Y705), ERK1/2 (Cell Signaling), CS, Histone H3 (Abcam) and β-actin (Sigma) overnight. All primary antibodies were used at a 1:1000 dilution unless otherwise stated. Blots were washed 3 times in TBST and incubated with either 1:10000 anti-rabbit or 1:10000 anti-mouse IRDye 800 secondary antibodies (Rockland) followed by imaging with a LiCOR Odyssey imaging system.

*2.3.5 Generation of shRNA-STAT3 and STAT3-constitutively active cell lines*

A doxycycline (Dox) induced Tet-ON system was used to generate short hairpin renilla control (shVEC) and shSTAT3 knockdown cell lines (100). Target STAT3 and renilla oligonucleotides were created (Integrated DNA Technologies) and cloned into the retroviral vector plasmid (TtRMPVIR). Virus was generated in 293T phoenix cells as per Swift, Lorens, Achacoso, & Nolan, 2001. Virion-containing supernatant was collected at 24 hours and 48 hours post transfection and was used to transduce DKO cells. Briefly, 1

mL of viral supernatant was incubated with DKO cells in the presence of 4 μg polybrene. After 2 hours, 1 mL of fresh media was added and cells were incubated at 37°C/5%$CO_2$ for 3 days. Cell culture media was completely replaced on day 2. On day 3, successfully transduced cells were analyzed via flow cytometry and individual cells were sorted based on green fluorescent protein (GFP) expression into single wells of a 96 well plates using a BD Influx™ Cell Sorter (BD Biosciences). After 10 days, putative shRNA-transduced DKO clones were further screened based on expression of red fluorescent protein (RFP) following 10nM Dox addition using a Guava EasyCyte Flow Cytometer (Guava Technologies), which was indicative of an active plasmid. Confirmed clones were expanded and STAT3 knockdown efficiency was assessed by immunoblotting as described.

A constitutive STAT3-expressing cell line was generated by transfection with the MSCV-Thy1.1-STAT3 plasmid (kindly provided by Dr. Mark Kaplan). This is a constitutively dimerizeable STAT3 was created by substituting cysteine residues for specific amino acids within the SH2 domain of STAT3. Viral propagation and transduction were performed as described above. Transduced DKO cells were single cell sorted into 96 well plates based on positive Thy1.1 expression and subsequently expanded for downstream analyses.

*2.3.6 Statistical analysis*

Unless otherwise indicated, statistical analyses were determined using one-way ANOVA plus a Dunnet post-test. All statistical calculations were completed using GraphPad 6.0 software, and p values $<0.05$ were considered significant.

## 2.4 Results

*2.4.1 Pharmacological inhibition of STAT3 supresses murine CD8+ T cell and human PBMC growth and proliferation following T cell receptor stimulation*

Preliminary data proposed an important role for STAT3 during the transition from a resting to a growth state. However, these experiments were performed in an IL-3 dependent cell line, suggesting a potential signaling bias towards the JAK/STAT (102). Thus additional experiments were performed to assess STAT3's importance in alternative models. Naïve T cells are known to be found in a resting state, indicative of decreased signaling and metabolic activity (103). During the induction of an immune response, T cells become highly proliferative following stimulation of the T cell receptor (TCR). This transition from a resting to an activated state provides an ideal model for studying the initiation of cell growth.

Naïve CD8+ T cells were isolated from murine splenocytes and stimulated *in vitro* with anti-CD3/anti-CD28 and IL-2 in the presence or absence of the STAT3 inhibitor WP1066. As expected, cells stimulated in the presence of WP1066 showed decreased expression of STAT3 and CS (Figures 7A). In addition, treatment with WP1066 also suppressed initial cell growth and proliferation following stimulation (Figures 7B and 7C). These results are similar to our previous findings, which demonstrated that DKO cells treated with WP1066 at the time of IL-3 readdition led to reduced STAT3 and CS levels, as well as reduced cell growth and proliferative capacity (Figures 4B, 4C and 6C).

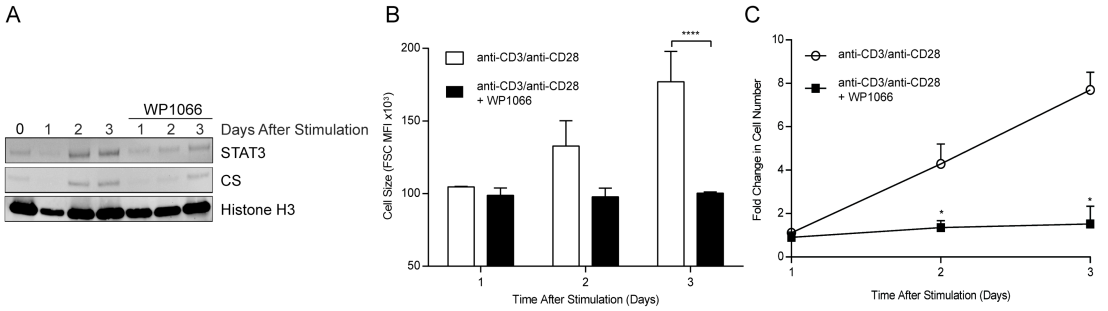


**Figure 7. Inhibition of STAT3 suppresses initial cell growth and proliferation in murine CD8+ T cells**. CD8+ T cells were stimulated with plate bound anti-CD3/anti-CD28 with IL-2 in the presence or absence of WP1066. (A) A representative immunoblot analysis (n=3) of STAT3 and CS. Histone H3 served as a loading control. (B) Cell size was measured by forward scatter, and (C) fold change in cell number was determined by 123count eBeads on the flow cytometer. Graphs show average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, *p<0.05, ****p < 0.0001).

To confirm our findings in an additional model, healthy donor PMBCs were activated *in vitro* with the mitogen phytohaemagglutinin (PHA) and IL-2 in the presence or absence of WP1066. Similar to the results observed with DKO and murine CD8+ T cells, STAT3 inhibition suppressed initial cell growth and proliferation of human PBMCs following PHA stimulation (Figures 8A and 8B). These experiments support the findings that STAT3 is essential for initial cell growth and proliferation and that this occurrence is not restricted to our DKO model system.

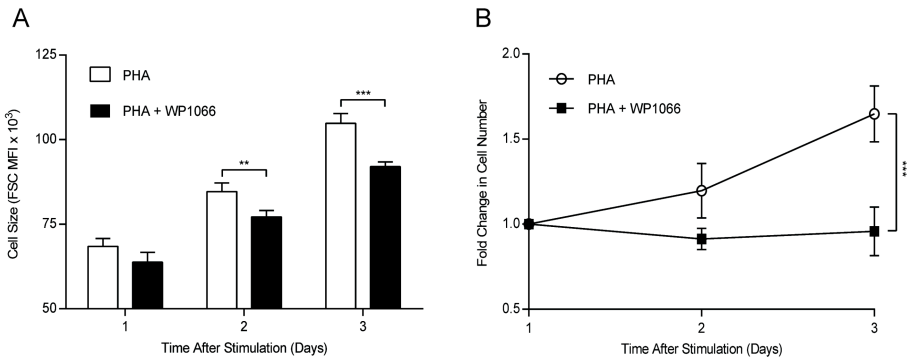


**Figure 8. Inhibition of STAT3 supresses initial cell growth and proliferation in human PBMCs**. Human PBMCs were stimulated with PHA and IL-2 in the presence or absence of WP1066. (A) Cell size was measured by forward scatter and (B) fold change in cell number was determined by 123count eBeads on the flow cytometer. Graphs show average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, **p<0.01, ***p < 0.001).

### *2.4.2 STAT3 knockdown suppresses citrate synthase expression, cell growth and proliferation in response to growth factors*

The importance of STAT3 during the initiation of cell growth has been demonstrated thus far with a pharmacological inhibitor in multiple cell lines. Thus to examine the specific effects of STAT3 on both cell size and CS expression, I generated an inducible shRNA-STAT3 DKO cell line. shRNA-STAT3 and shRNA-VEC control DKO cells were cultured in the absence of IL-3 for 14 days. Two days prior to IL-3 restimulation, STAT3 knockdown was induced by the addition of doxycycline (Dox). The readdition of IL-3 to control DKO cells (shRNA-VEC) resulted in an increase in both STAT3 and CS expression by 48 hours (Figure 9A). In contrast, STAT3 knockdown (shRNA-STAT3) not only inhibited STAT3 protein expression, but also prevented the recovery of CS protein levels following IL-3 readdition (Figure 9A). Furthermore, a significant block in the production of total intracellular citrate was observed as a result of

STAT3 knockdown 24 hours after IL-3 readdition (Figure 9B). This reduced expression of CS and decreased level of intracellular citrate is consistent with a loss of metabolic control by STAT3, as three independent shRNA-STAT3 knockdown clones were unable to regain their cell size between 6 and 24 hours following IL-3 readdition (Figure 9C). Moreover, unlike the control cells, which expanded almost 3.5-fold during this time, there was no increase in cell number in the shRNA-STAT3 expressing cells in response to IL-3 readdition (Figure 9D). These results are consistent with that observed using the STAT3 inhibitor, and suggest that STAT3 is directly involved in mediating the initiation of cell growth through effects on CS levels.

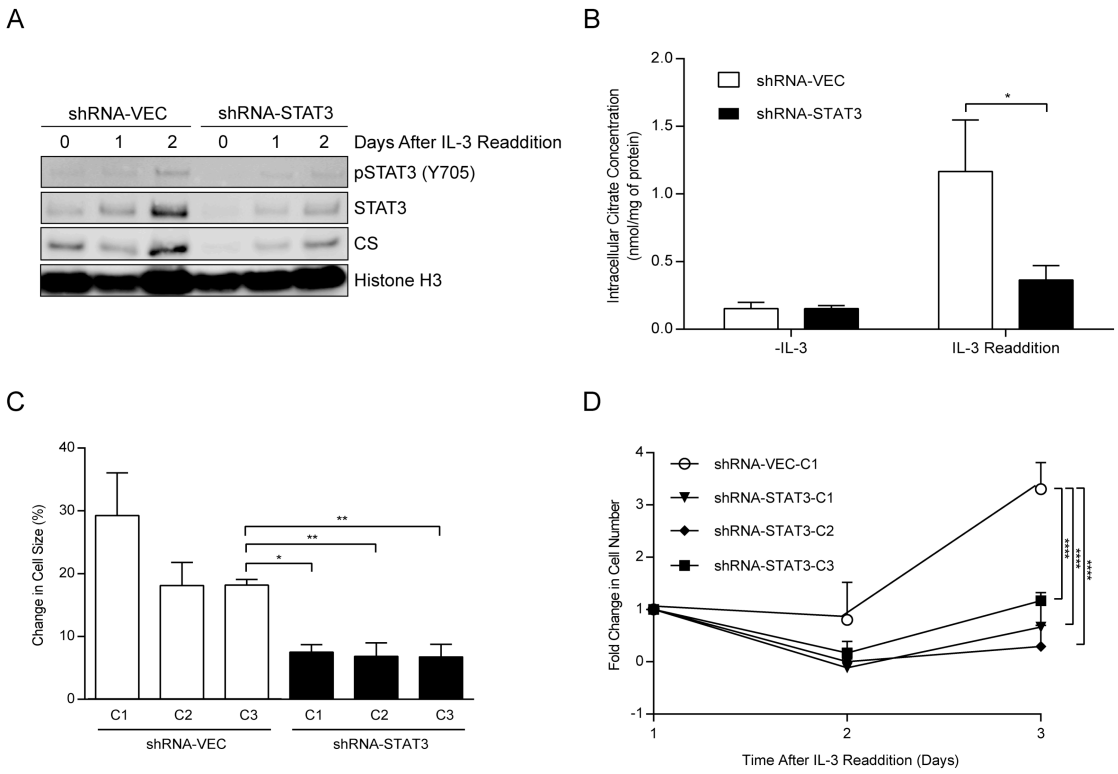


**Figure 9. STAT3 knockdown supresses CS and citrate levels leading to loss in initial cell growth and proliferation.** STAT3 knockdown (shRNA-STAT3) and vector control (shRNA-VEC) cells were starved of IL-3 for 14 days, with Dox addition on day 12 to induce shRNA. Upon IL-3 readdition, (A) immunoblot analysis of STAT3 and CS was performed (n=3). Histone H3 served as a loading control. (B) Citrate levels 24 hours after IL-3 readdition was examined, and (C) percent change in cell size from 6 to 24 hours in 3 individual clones (C1, C2, C3) post IL-3 readdition was measured by forward scatter. (D) Fold change in cell number was determined following 1-3 days post IL-3 readdition. Graph shows average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, *p<0.05, **p<0.01, ****p < 0.0001).

STAT3 is an essential transcription factor that regulates many cellular processes. STAT3 knockdown has been shown to inhibit growth of various cancers *in vitro* and *in vivo* (104–106). Therefore, shRNA-STAT3 and shRNA-VEC control cells were studied in the continued presence of IL-3 to determine the effects of STAT3 knockdown on cells that are already in an active proliferative state. Following two days of Dox treatment, STAT3 knockdown was confirmed by immunblot (Figure 10A). However, suppression of

STAT3 in the continued presence of IL-3 led to no change in CS expression, cell growth or proliferation (Figures 10A, 10B and 10C). These results suggest that STAT3 exerts its effects during the transition from a resting to cell growth state and does not affect cells that have already undergone this transition.

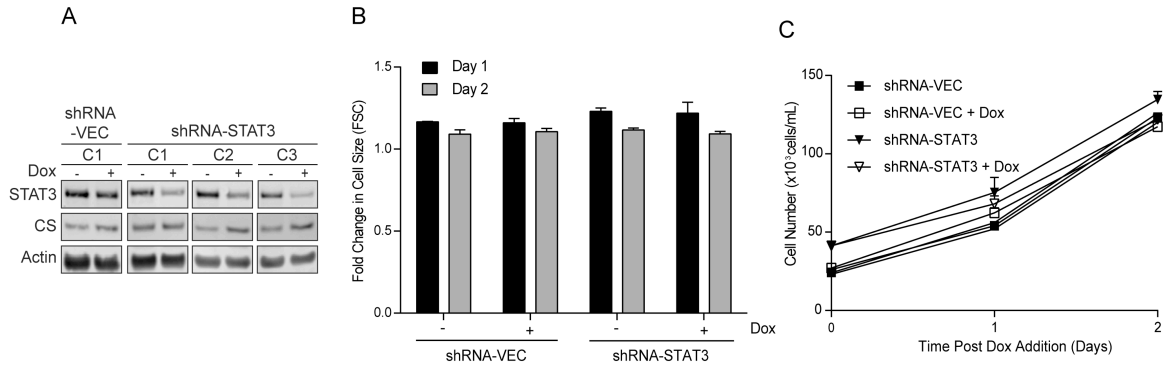


**Figure 10. In the continued presence of IL-3, STAT3 knockdown has no effect on CS, cell growth or proliferation.** (A) Immunoblot analysis of STAT3 and CS was performed on shRNA-VEC and shRNA-STAT3 cells cultured in the presence or absence of Dox for two days (n=3). (B) Fold change in cell size was measured by forward scatter 1 and 2 days post Dox treatment. (C) Cell number was determined following 1 and 2 days post Dox treatment. Graphs show average ± SD (n=3, one-way ANOVA plus a Dunnet post-test).

*2.4.3 STAT3 knockout supresses CS expression, initial cell growth and proliferation*

To further elucidate the importance of STAT3 during initial cell growth, naïve STAT3-knockout CD4+ T cells were isolated from the spleens of *CD4-Cre-STAT3$^{fl/fl}$* mice and cell growth and proliferation was examined after TCR activation (107). Naïve wild-type (CD4-Cre STAT3$^{+/+}$) and STAT3-knockout (CD4-Cre STAT3$^{-/-}$) CD4+ T cells were stimulated *in vitro* with anti-CD3/anti-CD28 and IL-2. Similar to previous findings using pharmacological inhibition and shRNA silencing, specific genetic ablation of

STAT3 in CD4+ T cells led to reduced CS expression, cell growth and proliferation (Figures 11A-11C).

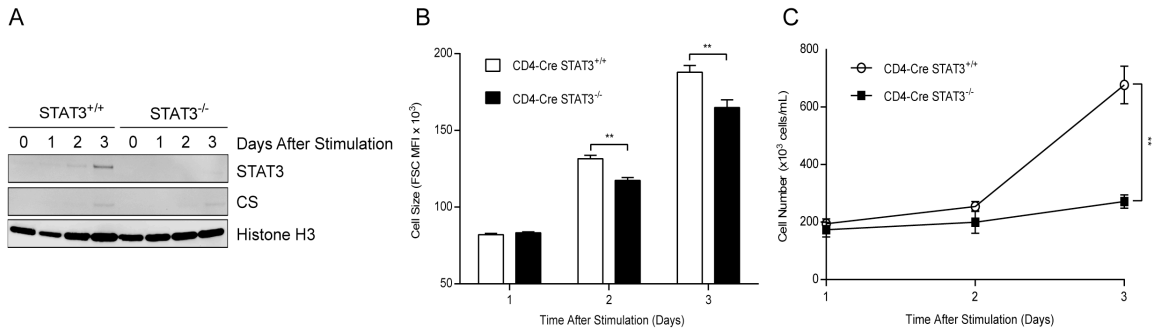


**Figure 11. Genetic ablation of STAT3 in CD4+ T cells supresses CS, initial cell growth and proliferation**. (A) Immunoblot analysis of STAT3 and CS following anti-CD3/anti-CD28 stimulation in CD4+ T cells harvested from *CD4-Cre STAT3*$^{+/+}$ and *CD4-Cre STAT3*$^{-/-}$ splenocytes (n=3). (B) CD4+ T cells were stimulated with anti-CD3/anti-CD28 and cell size and (C) cell number was examined. Graphs show average ± SEM (n=3, Student's *t*-test, **p<0.01).

*2.4.4 STAT3 promotes initial cell growth*

To determine whether STAT3 gain-of-function could promote initial cell growth following IL-3 readdition, a constitutively active STAT3 (CA-STAT3) DKO cell line was generated (Figure 12A). DKO cells expressing CA-STAT3 were cultured in the absence of IL-3 for 14 days. From 6 to 24 hours after IL-3 readdition, CA-STAT3 cells displayed a greater than 2.5-fold improvement in the recovery of initial cell size compared to vector controls (Figure 12B). Interestingly, CA-STAT3 cells were unable to recover mitochondrial membrane potential measured by TMRE post IL-3 readdition (Figure 12C). These results suggest that constitutive activation of STAT3 promotes the shunting of carbons out of the mitochondria for fatty acid synthesis in order to support initial cell growth, rather than allowing the TCA cycle to produce intermediates such as

NADH, and $FADH_2$, which instead promote proton transfer to the intermembrane space of the mitochondria creating mitochondrial membrane potential that is required for the synthesis of ATP. This data supports the link between the STAT3 signaling pathway and the metabolic pathways essential for initial cell growth.

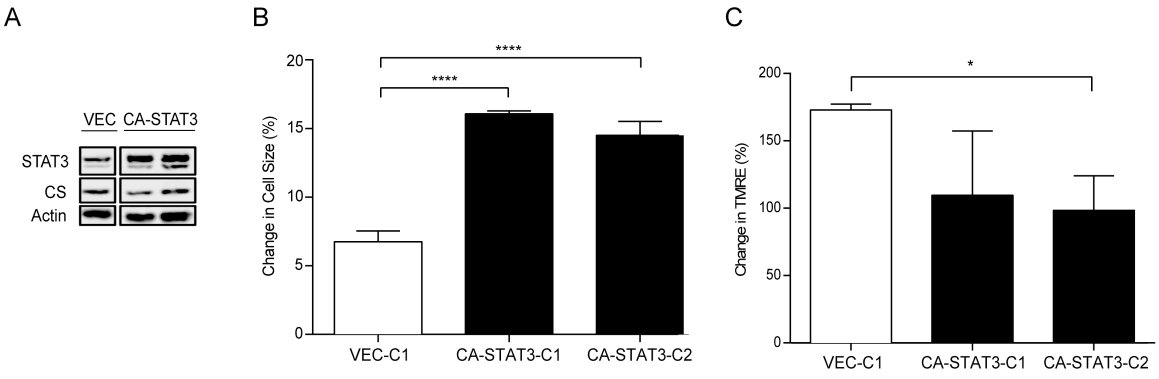


**Figure 12. Constitutively active STAT3 supports initial cell growth and supresses mitochondrial membrane potential.** Constitutively active STAT3 cells (CA-STAT3) or vector control (VEC) clones were starved of IL-3 for 14 days. Following IL-3 readdition, (A) Immunoblot of STAT3 and CS (n=3) (B) percent change in cell size from 6 to 24 hours measured by forward scatter (C) percent change in mitochondrial membrane potential measured by TMRE from 6 to 24 hours. Graph shows average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, *p<0.05, ****p<0.0001).

## 2.5 Discussion

Based on the above results, recovery from a resting state requires the expression of the transcription factor STAT3 to restore initial cell growth and proliferation following a stimulus. This role appears to be critical during the initial stages after growth factor stimulation of resting cells, as the suppression of STAT3 in exponentially growing cells had no impact on cell size or proliferation (Figures 10A-10C). This suggests that other signaling pathways may provide redundancy to ensure growth-dependent programs are sustained once cells have entered the cell cycle. Large-scale studies to determine the

signaling events after growth factor stimulation may shed light on the sequential pathways that are activated and how they contribute to metabolic reprogramming during the first phases of growth (108,109). However, as discussed previously, some of the cell's key signaling mediators, including STAT5, PI3K/AKT, and MAP/ERK are not involved in the recovery from IL-3 withdrawal, but rather their roles may become more important at later time points when cells have already proceeded into the cell cycle (Figure 5A).

STAT3 was originally identified as an acute phase response mediator that binds to IL-6-responsive cis-acting elements (110). Subsequent loss-of-function studies found that STAT3 was required for growth, proliferation and inhibition of apoptosis in response to *in vitro* stimulation with a broad range of cytokines and growth factors (75,78,111,112). These defects in growth and proliferative capacity are most clearly demonstrated in mice deficient for STAT3, but not other STAT members, whereby STAT3 knockout mice die during early embryogenesis. The wide range of cell types affected by lineage-dependent STAT3 signaling may also explain why it potentially plays a more prominent role in the coordination of metabolism than previously appreciated (113).

Although STAT5 is known to be activated by IL-3 (76), the appearance of phospho-STAT5 occurred subsequent to the observed changes in cell size (Figure 4A). This implies that STAT5 may have a more limited role during the specific stages of regulating the initial response to IL-3 readdition in this system. Even in the absence of STAT3 by genetic knockdown or chemical inhibition by WP1066, STAT5 was not sufficient to restore cell growth, mitochondrial membrane potential or proliferation. This

suggests that STAT5 does not play a significant part in the initiation of cell growth, and that this may be a STAT3 specific role.

The STAT3 inhibitor, WP1066, was shown to suppress increases in cell size in response to growth stimuli compared to the DMSO control, however despite the presence of the inhibitor there was still a slight increase in cell size over time. And yet, cells treated with WP1066 did not recover mitochondria membrane potential or proliferative capacity. These discrepancies may be perhaps explained by the following: first, it is possible that the concentration of WP1066 was not great enough to completely repress increases in cell size but was still able to prevent cell proliferation. This would suggest a type of metabolic threshold, whereby the generation of metabolites to support an increase in size is of primary importance, and the inability to reach this threshold leads to reduced production of downstream intermediates required for mitochondrial respiration is halted, ultimately preventing the regain of proliferative capacity.

Alternatively, this slight increase in cell size could be due to other transcriptional pathways that become activated in the event of STAT3 loss in order to rescue cell size in response to growth stimuli. This potential secondary pathway would support cell growth under the presence of the STAT3 inhibitor but is not sufficient to restore proliferation and mitochondrial membrane potential. This also could explain the slight increase in cell growth observed in the CD4 STAT3$^{-/-}$ cells (Figure 11B), which was not sufficient to restore proliferation. Thus although the loss of STAT3 does not completely ablate cell size, it's suppression during initial cell growth is sufficient to inhibit proliferation.

These results demonstrate the importance of STAT3 in the initiation of cell growth and proliferation. It also suggests that STAT3 may exert its regulatory effects by

modulating the enzyme citrate synthase. Together, these results may provide a potential novel mechanism linking signaling and metabolic pathways during the initiation of cell growth.

# Chapter 3: STAT3 Regulated Citrate Synthase is Essential for Initial Cell Growth

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J.J.L. and S.M. conceptualize and conducted the project. J.J.L. and S.M. contributed 5% and 95% respectively for the performed experiments. J.J.L., S.M., M.H., T.H. and J.Z. contributed 40%, 45%, 5%, 5%, 5% respectively in experimental design and scientific suggestions. All authors edited the manuscript.

## 3.1 Abstract

The coordination between cell signaling and metabolic pathways is crucial for cell division. However, the specific mechanism that links these two pathways during initial cell growth has yet to be defined. In Chapter 2, it was demonstrated that STAT3 plays an essential role during initial cell growth and proliferation, and that these effects corresponded to simultaneous alterations in CS levels. This observation led to the hypothesis that the effects of STAT3 during initial cell growth may be mediated through CS. Through shRNA knockdown of CS, I show that this enzyme is also essential for restoring cell size and proliferative capacity after growth factor stimulation, and that these effects were paralleled by reduced intracellular citrate. I demonstrate that STAT3 transcriptionally regulates CS expression during initial cell growth through two binding sites in the CS promoter, CS1 and CS2. Lastly, these effects were shown to be due to STAT3-dependent regulation of citrate levels via CS, as the defects in cell growth and proliferation during STAT3 inhibition could be restored by the addition of exogenous citrate.

## 3.2 Introduction

Previous data presented in Chapter 2 demonstrates an important role for STAT3 in the regulation of initial cell growth and proliferation. STAT3 suppression also resulted in the reduction of CS expression and citrate levels, key metabolic factors that support *de novo* fatty acid synthesis. These findings were consistent in all conditions and cell lines tested. This data suggests a potential mechanistic link between STAT3 and CS for initial cell growth.

In order for a cell to increase in size for division, the cell must synthesize *de novo* lipids for cell membrane construction and cell growth. To do so, the cell synthesizes glucose-derived citrate through the condensation reaction of oxaloacetate with acetyl-CoA by CS. The importance of CS can be supported in yeast, *E. coli* and *C. elegans*, as growth and division in response to nutrients has been shown to be dependent on CS (38–42). In addition, overexpression of CS has also been shown to increase the production of citrate and promote cell growth in *A. thaliana* and in *E. coli* (42,114). Thus CS may be an essential metabolic event for the production of citrate during the initiation of cell growth.

The regulation of the metabolic events involved in initial cell growth is relatively undefined. For gene expression regulation, transcription factors bind to specific consensus sequences found in the promoter of the gene that they regulate. The promoter region usually spans 100-1000 base pairs (bp) upstream of the transcription start site, however long-range gene regulation has been previously described (115). STAT3 has two putative consensus sequences in the promoter of the CS gene, CS1 and CS2. CS1 is a STAT3 binding site located 20,000 bp upstream of the CS gene. Until now, no studies have been conducted on this specific putative binding site. The CS2 binding site is located 54 bp upstream of the transcriptional start site of the CS gene. The CS2 binding site has been previously described as a target binding site of STAT3 during the development of the autoimmune disease colitis (116). Whether STAT3 binds and promotes either site during the initiation of cell growth is currently undescribed.

Here I study whether STAT3 binds to and promotes the transcription of CS through the CS1 or CS2 sites during initial cell growth by chromatin immunoprecipitation (ChIP) and reporter assays. Moreover, I determine the importance

of CS for initial cell growth and proliferation by shRNA knockdown. Lastly I attempt to rescue the loss in cell size and proliferation in shRNA-STAT3 and shRNA-CS cells by the addition of exogenous citrate.

## 3.3 Methods

### *3.3.1 Generation of shRNA cell lines*

A doxycycline (Dox) induced Tet-ON system was used to generate short hairpin renilla control (shVEC) and shCS knockdown cell lines (100). Target oligonucleotides were synthesized (Integrated DNA Technologies) and cloned into the retroviral vector plasmid (TtRMPVIR). Viral particles were generated in 293T phoenix cells as per Swift, Lorens, Achacoso, & Nolan, 2001. Virion-containing supernatant was collected at 24 hours and 48 hours post transfection and was used to transduce DKO cells. Briefly, 1 mL of viral supernatant was incubated with DKO cells in the presence of 4 μg polybrene. After 2 hours, 1 mL of fresh media was added and cells were incubated at 37°C/5%$CO_2$ for 3 days. Cell culture media was completely replenished on day 2. On day 3, cells that were successfully transduced were analyzed via flow cytometry. Cells were sorted based on green fluorescent protein (GFP) expression into single wells of a 96 well plates using a BD Influx$^{TM}$ Cell Sorter (BD Biosciences). After 10 days, potential shRNA clones that grew in the 96 well plate were further screened based on expression of red fluorescent protein (RFP) following 10nM Dox addition using a Guava EasyCyte Flow Cytometer (Guava Technologies), which was indicative of an active plasmid. Selected clones were expanded and CS knockdown efficiency was assessed by immunoblotting as described.

*3.3.2 Chromatin immunoprecipitation (ChIP)*

Chromatin immunoprecipation was performed as described previously (117). Briefly, cells were treated for 10 min with 37% (weight/volume) formaldehyde and incubated at room temperature for 15 minutes. Samples were then sonicated with the following cycle: 4 cycles of 15, 1 second pulses with 2 min rest periods. ChIP grade IgG (Cell Signaling; 1uL), Histone H3 (Cell Signaling; 3 uL), STAT3 and STAT5 (Cell Signaling; 5uL) antibodies were incubated with samples at 4 °C overnight while rocking. Protein A beads were incubated with the samples for 1 hour at 4 °C with gentle rocking followed by DNA enrichment with chelex beads (Bio-Rad). For detection of targets, the following primer pairs were synthesized and used: CS1 F:CAATGGTCGAGGCCGGGGTG, R:GGCACGCAGCACATGGCCTA. CS2 F: AAAGCCAGGAGACCTTGATG, R: CCCAAACAAGAAACAAAGGAAGA.

*3.3.3 Luciferase assay*

The CS1 and CS2 binding sites were generated with gBlocks (IDT) and cloned into the pGL4.23 (Promega) vector containing a minimal promoter and a firefly luciferase. Each gBlock was cloned by SacI and EcoRV restriction sites on either end of the 250 bp gene fragment (Table 1). Mutant binding site constructs were generated by modifying 4 consecutive nucleotides in the STAT3 consensus binding sequence. Each positive construct containing the CS binding site was confirmed by standard Sanger Sequencing (Genscript).

| | |
|---|---|
| CS1-WT | GTACCTGAGCTCCCTCAAAGCACCTCACAACCCTCAAGTCTACCTA<br>CTTAAAGCTTCGCTTTCTCATTGATATTGGAGTCAAGTTTGTGGGTC<br>ATGGTCTGCAGAAGGACTTCCGGGTCATCAACCTCATGGTTCGGGC<br>AGGGCTTATTTCAGAGTGTGTTTTTTGTTGTTGTTGTCTTCCTTTGTT<br>TCTTGTTTGGGTTGATATCAAGATC |
| CS1-MT | GTACCTGAGCTCCCTCAAAGCACCTCACAACCCTCAAGTCTACCTA<br>CTTAAAGCTTCGCTTTCTCATTGATATTGGAGTCAAGTTTGTGGGTC<br>ATGGTCTGCAGAAGGACgcgtGGGTCATCAACCTCATGGTTCGGGCA<br>GGGCTTATTTCAGAGTGTGTTTTTTGTTGTTGTTGTCTTCCTTTGTTT<br>CTTGTTTGGGTTGATATCAAGATC |
| CS2-WT | GGTACCTGAGCTCCCCCTCAGCCGCCTCCTTTCAACCTTGTCCGCC<br>GGTTGGCGCGGTCTCCATCACAGCGGCGACCGCTCCTGTTGCAGCT<br>GTAGCTCTCTCCCTTCGGTCCCTTCCCGCCAGGTCCCGGAGTTTCGC<br>CCGCCATGGCTCTACTCACTGCAGCAACCCGGCTCTTGGGAGCCAA<br>GGGATATCAAGATCT |
| CS2-MT | GGTACCTGAGCTCCCCCTCAGCCGCCTCCTTTCAACCTTGTCCGCC<br>GGTTGGCGCGGTCTCCATCACAGCGGCGACCGCTCCTGTTGCAGCT<br>GTAGCTCTCTCCCTTCGGTCCCTggaaGCCAGGTCCCGGAGTTTCGCC<br>CGCCATGGCTCTACTCACTGCAGCAACCCGGCTCTTGGGAGCCAAG<br>GGATATCAAGATCT |

**Table 1.** Oligonucleotides ordered from gBlocks containing SacI and EcoRV restriction sites (green), and the wild-type (WT) or mutant (MT) CS1 and CS2 binding sites (yellow). Exact mutation made is in lower case letters.

Single transfection (10 µg plasmid) and co-transfections with a renilla control luciferase reporter (pRLK) (3 µg) were performed in DKO cells by electroporation with Gene Pulser Xcell (Bio-Rad) 250 V and 960 µF. Transfected DKO cells were incubated in complete media containing IL-3 for 2 days before a 14 day IL-3 withdrawal. Whole cell lysates were collected 6 hours after IL-3 readdition and luciferase assays were conducted following the manufacturer's protocol (Promega). EnVison 2104 multilabel reader (PerkinElmer) was used to measure the expression of firefly and renilla luciferase activity.

*3.3.4 Cell culture and citrate rescue experiments*

The cell lines used for the majority of the experiments were an IL-3 dependent $Bax^{-/-}$ $Bak^{-/-}$ hematopoietic cells (DKO). DKO cells were cultured and starved as described in Chapter 2 with STAT3 or CS knockdown being induced by Dox on day 12 of the 14 day starve. At the time of IL-3 readdition, DKO cells were centrifuged and the media was replaced with fresh complete media containing IL-3 with or without 22mM sodium citrate.

## 3.4 Results

*3.4.1 STAT3 directly regulates citrate synthase during IL-3 recovery*

As shown in Chapter 1 and 2, pharmacological or genetic inhibition of STAT3 suppresses IL-3-mediated CS expression and initial cell growth and proliferation. To examine whether CS is a direct transcriptional target of STAT3, two STAT3 binding sites found in the CS promoter by ChIP-seq were analyzed. The first site is located 20 kbps upstream from the CS gene (CS1) and a second site contained a partial STAT3 consensus sequence 54 bps downstream of the beginning of the CS gene (CS2) that has been previously described (116). Next, chromatin immunoprecipitation (ChIP) was conducted to determine whether STAT3 could bind to these sites in response to IL-3 readdition. DKO cells were cultured in the absence of IL-3 for 14 days and cell lysates were collected 4 hours after IL-3 readdition. In IL-3-deprived cells, there was no association of STAT3 or STAT5 to either the putative CS1 or CS2 sites. However, within 4 hours of IL-3 readdition, STAT3 bound to both the CS1 and CS2 binding sites (Figure 13A).

To determine if either of the putative STAT3 sites, CS1 or CS2, could promote gene transcription after IL-3 stimulation, a luciferase reporter containing the wild-type (WT) or a mutant (MT) STAT3 consensus sequence was generated for both CS1 and CS2 (Figure 13B). The constructs were transfected into DKO cells two days before IL-3 deprivation. After 14 days of IL-3 withdrawal, the DKO cells were restimulated with IL-3. Whole cell lysates were collected 6 hours after the readdition of IL-3 to assess luciferase expression. In response to IL-3 readdition, there was a 4-fold increase in luciferase activity for both the STAT3 WT-CS1 and -CS2 sites compared to cells transfected with STAT3 MT-CS1 or -CS2, or cells that contained the empty luciferase vector (Figures 13C and 13D).

Figure 13

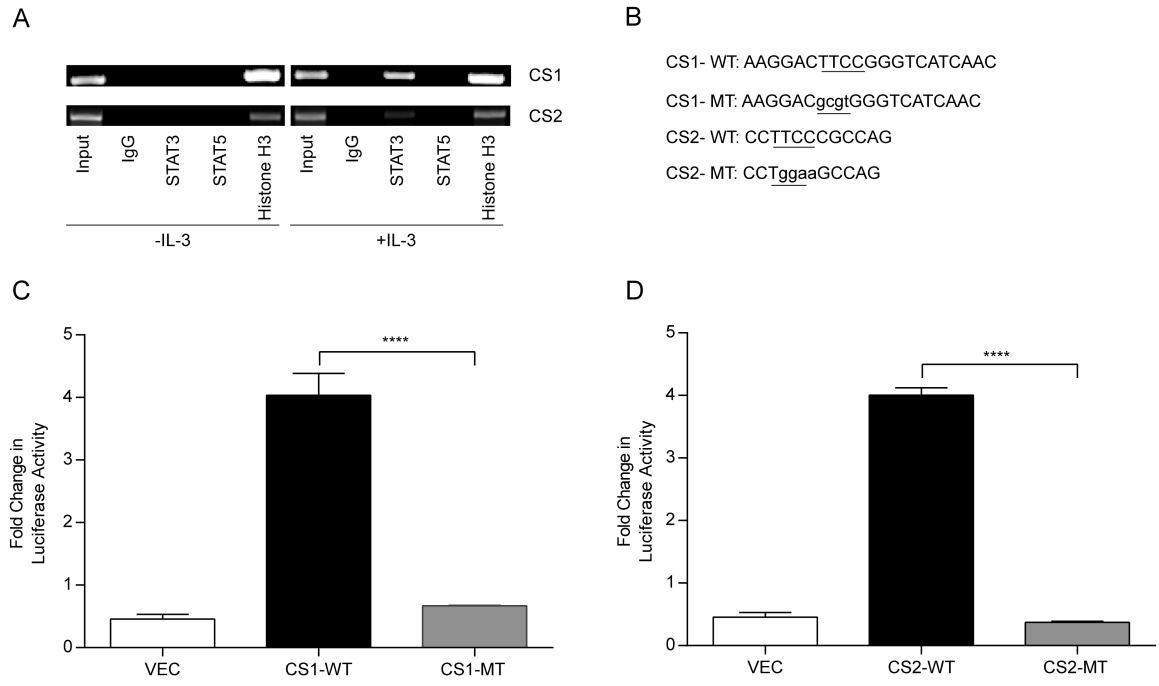


**Figure 13. STAT3 binds CS1 and CS2 to promote transcription of CS.** (A) DKO cells were starved of IL-3 for 14 days, followed by the readdition of IL-3 for 4 hours. ChIP analysis for IgG control, STAT3, STAT5 and H3 was performed. ChIP is representative of 2-3 individual experiments (B) STAT3 consensus sequence (underlined portion) of CS1 and CS2, and their respective mutants, used for the luciferase assays. (C) Fold change in luciferase activity for CS1-WT and CS2-MT 4 hours after IL-3 readdition.

(D) Fold change in luciferase activity for CS2-WT and CS2-MT 4 hours after IL-3 readdition. Graph shows average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, ****$p<0.0001$).

*3.4.2 Loss of CS expression impairs recovery of cell growth and proliferation*

The enzymatic product of CS, citrate, can remain in the mitochondria for subsequent oxidation or be exported to the cytosol for fatty acid biosynthesis (7,31). However, the above data suggests that in the absence of CS, a decrease in glucose-derived citrate would result in a significant impairment in cell growth. To test this, cell growth was assessed in IL-3 deprived DKO cells expressing an inducible shRNA-CS or control shRNA-VEC. Cells were deprived of IL-3 for 14 days and two days prior to IL-3 readdition, CS knockdown was induced by Dox treatment. Two days after Dox addition, IL-3 was reintroduced and immunoblots were performed to assess CS expression. Compared to the control cells, cells expressing the shRNA-CS showed a significant reduction in CS expression after IL-3 readdition (Figure 14A). During this time course, total intracellular citrate levels were significantly suppressed in the shRNA-CS clone 24 hours after IL-3 readdition (Figure 14B). The loss in CS expression and citrate levels resulted in a dramatic impairment in the recovery of cell size and proliferation following as shRNA-CS cells remained smaller and did not increase in cell number compared to the vector control (Figures 14C and 14D).

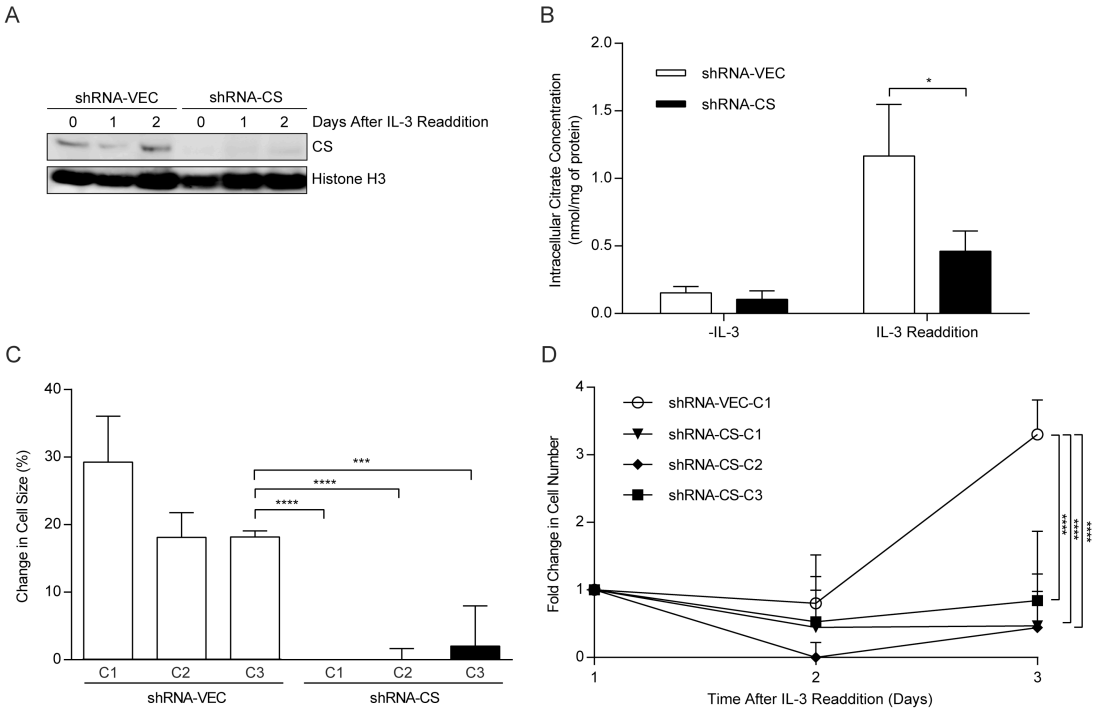


**Figure 14. CS knockdown suppresses citrate levels leading to loss in initial cell growth and proliferation.** CS knockdown (shRNA-CS) and vector control (shRNA-VEC) cells were starved of IL-3 for 14 days, with Dox addition on day 12 to induce shRNA. Upon IL-3 readdition, (A) immunoblot analysis of CS was performed (n=3). Histone H3 served as a loading control. (B) Citrate levels 24 hours after IL-3 readdition was examined, and (C) percent change in cell size from 6 to 24 hours post IL-3 readdition was measured by forward scatter. (D) Fold change in cell number was determined following 1-3 days post IL-3 readdition. Graph shows average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, *$p<0.05$, ***$p<0.001$, ****$p < 0.0001$).

The role of CS in an active proliferating state is unclear as CS has been demonstrated to have a variable role in proliferation in different cancer cell lines (118,119). Thus shRNA-CS and shRNA-VEC control cells were studied in the continued presence of IL-3 to determine the effects of CS knockdown in an active proliferative state. Following two days of Dox treatment, CS knockdown was confirmed by

immunoblot (Figure 15A). The suppression of CS in the continued presence of IL-3 led to no difference in cell growth or proliferation (Figures 15B and 15C).

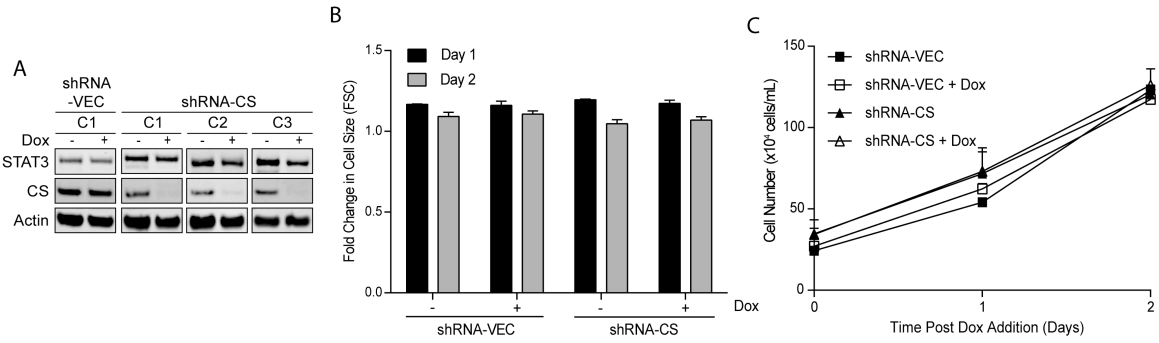


**Figure 15. In dividing cells, CS knockdown has no effect on cell growth and proliferation.** (A) Immunoblot analysis of CS was performed on shRNA-VEC and shRNA-CS cells cultured in the presence or absence of Dox for two days (n=3). (B) Fold change in cell size was measured by forward scatter 1 and 2 days post Dox treatment. (C) Cell number was determined following 1 and 2 days post Dox treatment. Graphs show average ± SD (n=3, one-way ANOVA plus a Dunnet post-test).

*3.4.3 Loss in cell growth and proliferation by STAT3 or CS knockdown can be rescued by exogenous citrate*

The above results show that both STAT3 and CS are essential for initial cell growth and proliferation. Results also suggest that STAT3 is involved in the transcriptional regulation of CS and that loss of STAT3 results in reduced CS expression and citrate levels during IL-3 restimulation. To support these findings, I attempted to rescue the loss in cell size and proliferation seen in the shRNA-STAT3 and shRNA-CS cell lines with exogenous citrate. Knockdown cell lines were cultured in the absence of IL-3 for 14 days. At the time of IL-3 readdition, cells were grown in the presence or absence of citrate. The addition of exogenous citrate to shRNA-STAT3 cells seemed to somewhat rescue cell size and proliferation (Figures 16A and 16B). Similarly, in the

shRNA-CS cell lines, citrate fully restored cell size and partially rescued proliferation (Figures 16C and 16D).

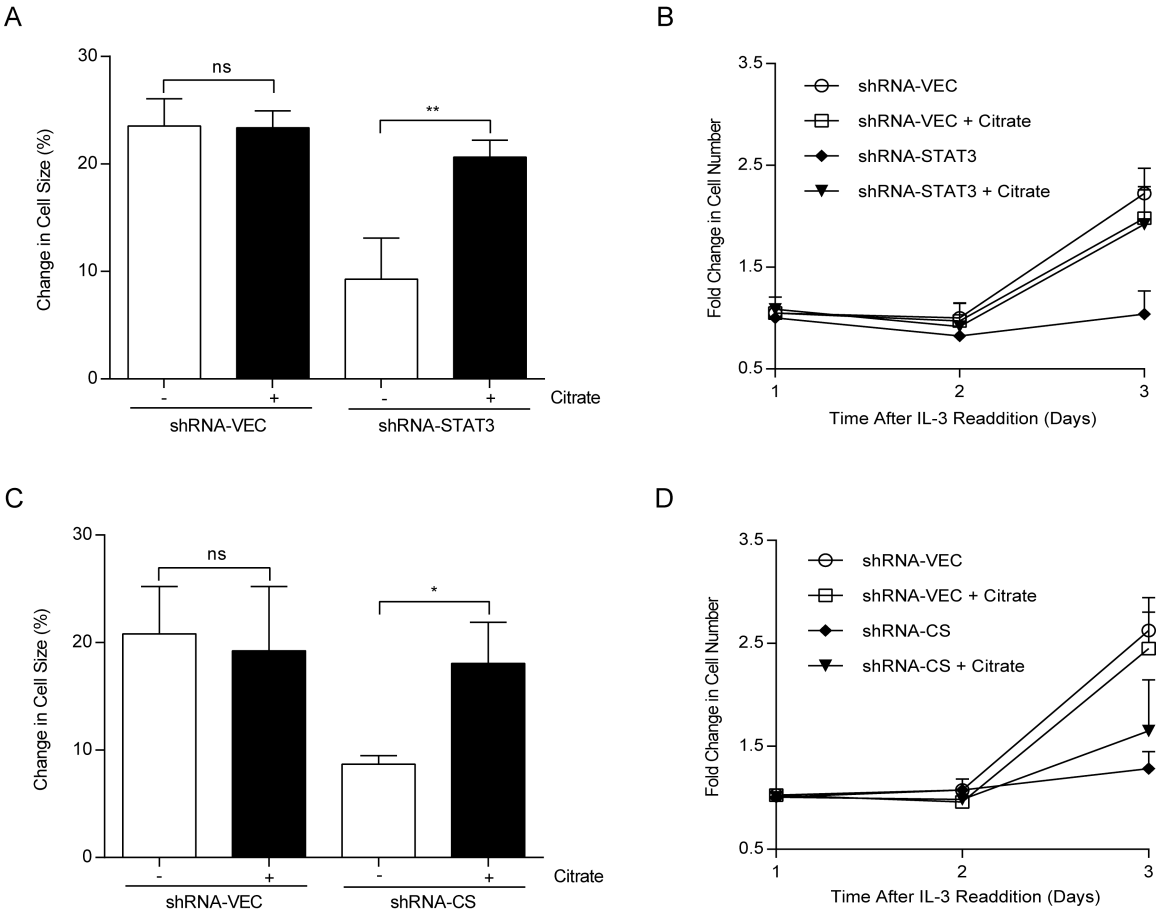


**Figure 16. Loss in cell growth and proliferation by shSTAT3 and shCS can be rescued by exogenous citrate.** Knockdown and vector control cells were starved of IL-3 for 14 days with Dox addition on day 12. Analysis were performed upon IL-3 readdition in the presence or absence of exogenous citrate: (A,C) percent change in cell size from 6 to 24 hours measured by forward scatter (B,D) fold change in cell number was determine 1-3 days after IL-3 readdtion. Graph shows average ± SD (n=3, one-way ANOVA plus a Dunnet post-test, *$p<0.05$, **$p<0.01$).

## 3.5 Discussion

Based on the above results, STAT3 transcriptionally regulates CS following growth factor readdition. This regulation appears to be specific to the initiation of cell growth as STAT3 does not bind to the CS1 or CS2 in a resting state. Similar to the shSTAT3 results found in Chapter 2, shCS led to suppressed intracellular levels of citrate, initial cell growth and proliferation. Interestingly the loss in initial cell growth and proliferation in both shSTAT3 and shCS cells could be rescued by the addition of exogenous citrate. Taken together, this data supports a mechanism whereby STAT3 transcriptionally regulates CS for the production of citrate to support initial cell growth and proliferation.

Citrate synthase is regulated by several key intracellular metabolites including acetyl-CoA and oxaloacetate, as well as ATP, NADH, citrate and succinyl-CoA (120,121). The TCA cycle and the electron transport chain have a harmonious relationship in that they each depend on the other to function appropriately. Thus it is possible that the loss in CS could affect the function of cellular respiration leading to the inability to initiate proliferation. However previous findings in our laboratory observed that upon IL-3 readdition in DKO cells, that mitochondrial membrane potential does not significantly increase until 3 days post readdition. This suggests that electron transport is not an essential process following IL-3 readdition and that it is more important after the cell increases in size and prepares for cell division. However, further experiments are required to support that of the loss of CS on the electron transport chain during initial cell growth.

It was shown in this chapter that the loss in initial cell growth from 6 to 24 hours in shSTAT3 and shCS cells was rescued by exogenous citrate. However, some would speculate this finding, as citrate is a known allosteric inhibitor of phosphofructokinase, a glycolytic enzyme. The inhibition of glycolysis impairs the utilization of glucose for the production of other critical cell building blocks as well as *de novo* citrate for fatty acid synthesis. On the contrary, this inhibition of glycolysis would promote the utilization of the pentose phosphate pathway that supports the synthesis of NADPH, a coenzyme required for fatty acid synthesis. Interestingly, the product of the pentose phosphate pathway will reenter glycolysis downstream of the phosphofructokinase reaction. Thus the addition of exogenous citrate will promote upstream metabolic events that will support fatty acid synthesis.

Collectively, this study defines a previously undescribed metabolic function of STAT3 as a key transcription factor that is directly involved in the regulation of the TCA cycle enzyme citrate synthase. This regulation is important for the metabolic control of cells in response to growth factors and may help to explain why unlike other STAT deficient mice, constitutive and tissue-specific STAT3 knockout mice exhibit defects in proliferation and survival (122).

# Chapter 4: Concluding Remarks

## 4.1 Chapter summaries and discussion

The overall objective of this thesis was to better understand the signaling and metabolic pathways required for the transition from a resting state, to a state of cell growth. More specifically, I investigated the role of STAT3 and its influence on the metabolic enzyme CS during initial cell growth. The transcriptional role of STAT3 on CS provides a previously un-described understanding of initial cell growth.

Previous experiments in the laboratory demonstrated that small molecule inhibition of STAT3 prevents initiation of cell growth and proliferation following growth factor readdition. In Chapter 2, I further investigated the role of STAT3 during initial cell growth. First, I confirmed the importance of STAT3 on initiation of cell growth in other cell types by inhibiting STAT3 in naive murine CD8+ T cells and human PMBCs. I next investigated the mechanism by which STAT3 suppresses initial cell growth and proliferation. Metabolic analysis of shSTAT3 cells during initial cell growth revealed that two key components for fatty acid synthesis, CS and citrate levels were suppressed. Fatty acid synthesis is essential for the production of lipids to support cell membrane construction for cell growth; therefore these findings support a potential link between STAT3 and CS during initial cell growth.

Alternative to the specific regulation of CS by STAT3, STAT3 could regulate other metabolic events upstream of CS. For example, STAT3 has been described to regulate the expression of GLUT1, a glucose transporter (82). Glucose transport is crucial for the synthesis of cell building blocks like lipids for cell membrane construction. Thus, inhibition of STAT3 could result in the suppression of glucose transport, glycolysis and

thus carbon-derived citrate for fatty acid synthesis. If this were true, the loss in mitochondrial membrane potential observed following STAT3 inhibition would be rescued by the exogenous addition of a glycolytic intermediate. However, previous experiments shown in the introduction established that pyruvate, the final product of glycolysis, was unable to rescue the loss mitochondrial membrane potential. Instead, the addition of a-ketoglutarate, an intermediate downstream of CS in the TCA cycle, rescued the loss in membrane potential due to STAT3 inhibition. This indicates that although STAT3 may influence other metabolic reactions during initial cell growth, the metabolic events upstream of CS seem relatively unaffected by loss of STAT3.

Alternative to STAT3, other signaling pathways could be involved in initial cell growth. Analysis of AKT and ERK indicated that they are not involved in the recovery from IL-3 withdrawal as their expression appears only after cells have initiated cell growth. Other kinases and transcription factors including, mTOR, Myc and E2F could be involved in initial cell growth in a STAT3-dependent or independent manner due to their well-known involvement in cell cycle regulation and cell metabolism (123–125). The protein kinase mTOR (mammalian target of rapamycin), has been previously described to be involved in cell cycle progression and the initiation of cell division (126). Interestingly, mTOR has been shown to support STAT3 phosphorylation to promote cell differentiation, suggesting a potential role for mTOR in the activation of STAT3 for initial cell growth (127–129). In addition, the transcription factors Myc and E2F have both been described to be involved in the initiation of proliferation (130,131). Conversely, some research has proposed a regulatory role for STAT3 on Myc and E2F during exit from quiescence (132,133). Further studies are necessary to determine the

signaling events that occur after initial cell growth when transitioning to a proliferative state.

In Chapter 3, I investigated the mechanism of transcriptional regulation of CS by STAT3 during initial cell growth as Chapter 2 supported a potential link between these two pathways. Chromatin immunoprecipitation analyses demonstrate that STAT3 binds two regions of the CS promoter, CS1 and CS2, specifically during initial cell growth. Furthermore, a luciferase reporter assay demonstrated that both CS1 and CS2 promote transcription during initial cell growth. I also found that citrate levels, cell growth and proliferation were suppressed in shCS cells, similar to what was observed in shSTAT3 cells in Chapter 2. This provides a mechanism for initial cell growth, whereby STAT3 transcriptionally regulates CS by binding to the CS promoter, to promote the synthesis of citrate for initial cell growth.

Luciferase reporter assays demonstrated that both CS binding sites, CS1 and CS2, had high activity specifically during initial cell growth. One key question that remains from Chapter 3 is whether either or both binding sites are essential for initial cell growth. For many genes, regulatory promoters tend to be located in close proximity to the gene of interest. The CS2 binding site is found 54bp from the transcriptional start site of CS and therefore its ability to regulate CS expression is very likely. Importantly, the CS2 binding site has been previously describe to be a target binding site for STAT3 during the induction of colitis in mice (116). Aside from this, there are no other descriptions of the CS2 STAT3 binding site to my knowledge. However whether CS2 is necessary for initial cell growth is unknown, thus further examination of its importance is required.

Many genes also require multiple cis-acting distant genomic elements for spatiotemporally correct expression, called enhancers (134). Enhancers do not act on the promoter itself, but are bound to activator proteins and can be found thousands of base pairs up or downstream of the genes that they regulate (115). The CS1 binding site is located 20,000bp upstream from CS in the PAN2 (Poly(A) Specific Ribonucelease subunit) gene. Long distance enhancers have been previously described to be found in the exogenic regions of unrelated genes (135–137). Although long distance regulation of CS expression through the CS1 binding site is plausible, the analysis of its importance becomes rather convoluted due to its location in PAN2. It is possible that PAN2 is being regulated through STAT3 during initial cell growth and that the CS1 binding site is unrelated to CS expression. Alternatively, PAN2 may be involved in CS and citrate regulation as part of RNA metabolism and translational efficiency. Thus the importance of CS1 in the regulation of CS has not been fully elucidated and requires further examination.

It is also possible that both of the putative STAT3 binding sites are necessary or act in synergy for the transcription of CS. The synergistic enhancement of transcription through multiple binding sites has been described previously (138). Here, I found that both CS1 and CS2 were utilized during initial cell growth, indicating that the two binding sites could act synergistically. Specifically, I found that STAT3-mediated transcription of CS through CS1 and CS2 is limited to initial cell growth following growth factor readdition, as STAT3 does not bind CS1 or CS2 under resting conditions. This suggests a transient interaction between the two binding sites. Transient interactions have been previously described to by supported by specific chromatin folding (115). Interestingly, it

was recently found that resting yeast cells undergo reorganization of the genome whereby intrachromosomal associations increase at longer distances compared to growing yeast cells (139). This proposes that chromosome folding may be high when transitioning from a resting state to a state of cell growth. Although both CS binding sites are active, further studies are required to determine the spatial organization of the region spanning the promoter of the CS gene during initial cell growth.

## 4.2 Integrating concepts from Chapters 2 and 3

In Chapter 2 and 3, I demonstrated an important connection between the transcription factor STAT3 and the metabolic enzyme CS in initial cell growth. Each chapter establishes that suppression of STAT3 or CS results in the inability to recover from a resting state. Together, I demonstrate that the regulation of initial cell growth is mediated by the transcriptional regulation of CS by STAT3 to synthesize citrate for fatty acid synthesis.

The results from Chapters 2 and 3 led me to investigate the connection between STAT3 and CS further by rescuing the loss in cell size and proliferation observed in shSTAT3 and shCS cells with exogenous citrate. As shown in Chapter 3, I found that the addition of exogenous citrate rescued initial cell size and proliferation, indicating that the regulation of citrate production by STAT3 and CS is essential for initial cell growth. These results identified the importance of the condensation reaction between acetyl-CoA and oxaloacetate by CS for the production of citrate for fatty acid synthesis to support cell growth. Importantly, this data indicates that STAT3 mediates initial cell growth through CS as the effects of STAT3 knockdown can be overcome by the addition of

citrate. This connection between STAT3 and CS was further demonstrated by the ability of STAT3 to bind to the promoter of the CS gene during initial cell growth. These data indicate that there is a clear connection between STAT3 and CS during initial cell growth.

## 4.3 Future directions

The results presented in this thesis, support a potential mechanism for initial cell growth. Further research is required to fully elucidate the importance and potential link between CS1 and CS2. Furthermore, a better understanding of the relevance of this mechanism *in vivo* could provide an appreciation of it's therapeutic potential for targeting cells initiating cell growth, a transition commonly observed in autoimmune disease and tumor recurrence.

The importance of the CS1 and CS2 binding sites during the initiation of cell growth will be studied by direct genetic modification using the CRISPR/Cas system. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and the CRISPR-associated protein nuclease (Cas) is an experimental tool recently developed that utilizes the adaptive immune system in bacteria for eliminating invading genetic material for targeted genome editing in mammalian cells (140). This system will allow for selective deletion of 20 base pairs surrounding the CS1 or the CS2 binding site. This experiment will determine whether either or both binding sites are necessary for initial cell growth. A better understanding of the importance of these binding sites could provide a specific mechanism for targeting initial cell growth.

The ability of citrate to rescue cell growth in the absence of CS or STAT3 is very interesting. However, additional research to demonstrate that the exogenous citrate is in fact utilized for fatty acid synthesis is necessary. To demonstrate this, I suggest using an ATP citrate lyase (ACLY) inhibitor at the time of IL-3 readdition and citrate rescue. The metabolic enzyme ACLY is involved in the conversion of citrate to acetyl-CoA in the cytosol. Thus inhibition of ACLY would prevent fatty acid synthesis and cell growth, thus supporting that the exogenous citrate is in fact utilized for cell growth. Alternatively, I could carbon label citrate and trace the metabolic events that it undergoes. This experiment would more clearly support that citrate is utilized for fatty acid synthesis, as currently I have no data supporting that citrate is utilized for fatty acid synthesis during initial cell growth. Thus these experiments would further test the metabolic mechanism described in this thesis.

Furthermore, the importance of STAT3 regulation of CS during initial cell growth will be further elucidated *in vivo*. The citrate rescue experiments performed in Chapter 3 established a connection between STAT3 and CS, as the loss in cell growth and proliferation was rescued in both shSTAT3 and shCS cells by the addition of exogenous citrate. Thus recapitulating this experiment *in vivo* would further support the importance of CS regulation by STAT3 during initial cell growth. To do so, a previously described T cell transfer model will be utilized, whereby naïve T cells are adoptively transferred into mice lacking an immune system leading to the induction of the autoimmune disease colitis (116). Using this model, Dr. John O'Shea and colleagues found that adoptive transfer of $STAT3^{-/-}$ naïve T cells does not result in the development of colitis (116). This finding recapitulates my *in vitro* findings that STAT3 is essential for initial cell growth

and proliferation of naïve T cells. Thus to support my findings that STAT3 regulates CS during initial cell growth, I will adoptively transfer $STAT3^{-/-}$ naïve T cells into mice lacking an immune system, with and without a citrate high diet (141). I would expect that the citrate high diet would rescue the inability of STAT3 to support citrate production for cell growth, resulting in the development of colitis. This would be a very interesting experiment which would not only support the importance of the mechanism described in the thesis, but would also suggest a potential therapeutic target for autoimmunity.

### 4.3.1 Broader implications

The transition from a resting state to a state of cell growth is commonly observed in autoimmune diseases and tumor recurrence, thus the mechanism described in this thesis could denote a novel target for these pathological conditions. Both STAT3 and fatty acid synthesis have been identified separately to support the development of autoimmune diseases (116,142–144). To my knowledge a connection has yet to be made between STAT3 and fatty acid synthesis in the promotion of autoimmunity. While Both fatty acid synthase and STAT3 have been developing as a model targets for cancer therapy, as they promote tumor cell growth (32,105,145,146). Fatty acid synthase levels have also been described to be involved in tumor recurrence (147). A novel therapeutic aspect of the research shown here is its specificity to the CS1 and CS2 binding sites exclusively during the initiation of cell growth. Thus, this research could provide a promising target for autoimmune diseases and tumor recurrence.

In summary, the findings presented in this thesis have provided a previously un-described mechanism for initial cell growth. I hope that the research on this project

continues in hopes of the discovering a novel target that specifically inhibits initial cell growth.

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# Appendix A: Abbreviations

| | |
|---|---|
| ACLY | ATP citrate lyase |
| AKT | protein kinase B |
| ATP | adenosine triphosphate |
| Cas | CRISPR-associated protein nuclease |
| ChIP | chromatin immunoprecipitation |
| CoA | coenzyme A |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| CS | citrate synthase |
| Dox | doxycycline |
| FADH | flavin adenine dinucleotide |
| FSC | forward scatter |
| GF | growth factor |
| GFP | green fluorescent protein |
| GLUT1 | glucose transporter 1 |
| Grb2 | growth factor receptor-bound protein 2 |
| JAK | janus kinase |
| MAPK | mitogen-activated protein kinase |
| MOP | dimethyl-2-oxoglutarate |
| MP | methyl-pyruvate |
| mRNA | messenger RNA |
| mTOR | mammalian target of rapamycin |
| NADH | nicotinamide adenine dinucleotide |

| | |
|---|---|
| PAN2 | poly(A) specific ribonuclease subunit |
| PBS | phosphate-buffered saline |
| PI3K | phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PHA | phytohaemagglutinin |
| PBMC | peripheral blood mononuclear cells |
| RFP | red fluorescent protein |
| STAT | signal transducer activator of transcription |
| SEM | standard error of the mean |
| SD | standard deviation |
| SLC25A1 | tricaboxylate carrier |
| Sos | son of sevenless |
| TCA | tricarboxylic acid |
| TCR | T cell receptor |
| TMRE | tetramethylrhodamine, ethyl ester |
| α-KG | alpha-ketoglutarate |