The Role of SIRT5 and Protein Succinylation in Regulating Cardiac Function and Metabolism

by

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Howard Rockman

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Sirtuin 5 (SIRT5) is one of three mitochondrial proteins that belongs to the sirtuin family of NAD⁺-dependent deacylases. Mitochondrial sirtuins (SIRT3-5) control metabolism in physiological and pathophysiological conditions by their deacylation activity. SIRT5 possesses demalonylase, desuccinylase, and deglutarylase activity. While the enzymatic activity of SIRT5 has been well characterized, the physiological role of SIRT5 is less understood. Recent evidence suggests that SIRT5 may have a role in responding to cardiac stress. Given that succinylation is abundant in the SIRT5KO heart, it is important to understand the role of SIRT5 mediated desuccinylation in the heart. Since it appears that there are no defects in cardiac function in SIRT5KO mice under basal conditions, I hypothesized that a stress would be required to determine a protective effect of SIRT5 in the heart. Additionally, I hypothesized that multiple sites of lysine succinylation would contribute to the overall phenotype observed.

To address this hypothesis, genetic mouse models were exposed to a well characterized model of pressure overload induced cardiac hypertrophy—transverse aortic constriction (TAC). Two main mouse models were used: 1) a whole body SIRT5KO mouse and 2) a cardiomyocyte tamoxifen-inducible heart specific SIRT5KO mouse. In order to characterize cardiac structure and function following TAC, the methods of serial echocardiogram and pressure volume loops with inferior vena cava
suppression were used. Mechanistic studies included metabolomic and succinyl-proteomic profiling of heart samples. Additionally, Western Blot and RT-qPCR were used to further gain mechanistic insight.

Chapter 3 of this dissertation characterizes the response of the whole body SIRT5KO mouse to pressure overload induced hypertrophy compared to littermate controls. We found that exposure to chronic TAC significantly increases mortality in SIRT5KO mice. Analysis of cardiac morphology and function after 4 weeks of TAC shows that SIRT5KO that have survived to this point have similar cardiac morphology and function compared to WT TAC mice. We predict that impaired oxidative metabolism is a major contributor to accelerated death in SIRT5KO mice.

To specifically investigate the role of SIRT5 in cardiomyocytes, a heart-specific, inducible SIRT5KO mouse was generated and exposed pressure overload via TAC surgery. We find that the phenotype of increased mortality in whole-body SIRT5KO mice is not recapitulated under the conditions tested in heart-specific SIRT5KO mice. However, the two genetic mouse models show differences in protein succinylation, leading us to perform succinyl-proteomics in this model. The results of this investigation are presented in Chapter 4. Collectively, the results of these studies provide new insight into the role of SIRT5 mediated desuccinylation in regulating cardiac function and metabolism.
Dedication

To Andrew and my family;
for your constant support and encouragement
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List of Abbreviations

36B4 – ribosomal protein, large, P0

4E-BP1 – translation initiation factor 4E binding protein 1

Acadl – acyl-coenzyme A dehydrogenase, long chain

Acadm – acyl-coenzyme A dehydrogenase, medium chain

Acadvl – acyl-coenzyme A dehydrogenase, very long chain

ACC – acetyl-CoA carboxylase

ACN – acetonitrile

Adb1 – adrenergic receptor, beta 1

Adb2 – adrenergic receptor, beta 2

AMPK – AMP (adenosine monophosphate)-activated protein kinase

Anf – natriuretic peptide, type A

ATP – adenosine triphosphate

BCA – bicinechonic acid

Bnp – natriuretic peptide, type B

CaMKII – Ca²⁺/calmodulin-dependent protein kinase II

Col1a1 – collagen, type I, alpha 1

Col3a1 – collagen, type III, alpha 1

Cpt1b – carnitine palmitoyltransferase 1b, muscle

DTT – dithiothreitol
EDD – end diastolic diameter
EDPVR – end diastolic pressure volume relationship
Emax – maximal elastance
ESD – end systolic diameter
ESPVR – end systolic pressure volume relationship
ETC – electron transport chain
E’max – maximum slope of quadratic EDPVR
FA – formic acid
FAO – fatty acid oxidation
FS – fractional shortening
Glut1 – solute carrier family 2 (facilitated glucose transporter), member 1
Glut4 – solute carrier family 2 (facilitated glucose transporter), member 4
IAM – iodoacetamide
IVSW – interventricular septum wall
Il6 – interleukin 6
Il10 – interleukin 10
KO – knockout
Ldha – lactate dehydrogenase
LVEDP – left ventricle end diastolic pressure
LVESP – left ventricle end systolic pressure
NA – nicotinic acid
NAD⁺ – nicotinamide adenine dinucleotide
NMN – nicotinamide mononucleotide
NNT – nicotinamide nucleotide transhydrogenase
NR – nicotinamide riboside
OXPHOS – oxidative phosphorylation
PCR – polymerase chain reaction
PCr – phosphocreatine
PDH – pyruvate dehydrogenase
Pdk4 – pyruvate dehydrogenase kinase, isoenzyme 4
Pgc1α – peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
PRSW – preload recruitable stroke work
PVA – pressure volume area
PW – posterior wall
Pparα – peroxisome proliferator activated receptor alpha
Ppard – peroxisome proliferator activated receptor delta
Rcan1 – regulator of calcineurin 1
RT-qPCR – real time quantitative polymerase chain reaction
SIRT1-7 – sirtuin 1-7
TAC – transverse aortic constriction
TCA – tricarboxylic acid cycle

TEAB – triethylammonium bicarbonate

TFA – trifluoroacetic acid

$V_0$ – volume intercept

WT – wild type

WTh – wall thickness
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1. Introduction

The sirtuins are a family of NAD\(^+\)-dependent enzymes that regulate gene
silencing, chromosome stability, and metabolism by removing acyl-lysine modifications
from a variety of proteins (Anderson et al., 2014). The SIR (silent information regulator)
family of genes were first discovered in *Saccharomyces cerevisiae* and were identified as
necessary for repressing translation of the silent mating type loci; indeed, inactivation of
any of these genes results in sterility (Ivy et al., 1986; Rine and Herskowitz, 1987). *Sir2*,
the only SIR gene conserved in higher eukaryotes, was shown to promote longevity in
*Saccharomyces cerevisiae* and spurred great interested in the field (Kaeberlein et al., 1999)
of Sir2-like proteins (sirtuins). The identification of Sir2 as an NAD\(^+\)-dependent
deacetylase initiated the idea that this class of proteins may serve to sense the energy or
nutrient status of cells (Imai et al., 2000).

The sirtuins are highly conserved from prokaryotes to mammals. In mammals,
seven sirtuins (SIRT1-7) occupy different cellular compartments. SIRT1, 6, and 7 reside
in the nucleus and nucleolus; SIRT2 resides primarily in the cytoplasm. Three sirtuins
(SIRT3, SIRT4, and SIRT5) are localized in mitochondria and are of particular interest
due to their role in regulating metabolism by removal of acyl-lysine post translational
modifications. The sirtuins are divided into classes based on the conservation of a core
domain that is approximately 250 amino acids (Frye, 2000) (Figure 1 A). Of the seven
mammalian sirtuins, SIRT1-3 are class I, SIRT4 is class II, SIRT5 is class III, and SIRT6-7
are class IV. An additional class (Class U) contains no mammalian sirtuins and is thought to be a precursor of Class I and Class IV sirtuins (Frye, 2006). It is thought that the seven mammalian sirtuins evolved from the engulfment of a Proteobacterium (from which mitochondria originated) containing a Class II and Class U sirtuin by an Archaean containing a Class III sirtuin (Frye, 2006). Originally, sirtuins were described as lysine-deacetylases. However, it is now clear that the acyl-lysine modifications that this family of proteins removes is broad and may reflect the classification of the sirtuins (Figure 1 B). For example, SIRT1-3 are Class I sirtuins and all possess strong lysine deacetylase activity (Hirschey, 2011). Given our interest in metabolic regulation, we further explore the enzymatic activities and physiological roles of the mitochondrial sirtuins.
Figure 1: Molecular phylogeny of the sirtuins.

(A) An unrooted tree diagram of a phylogenetic analysis of the conserved sirtuin core deacylase domain sequences, divided in Class I, II, III, IV, and U groups; classes I and IV are further divided into subclasses indicated by lowercase letters. (B) Schematic of conserved sirtuin core deacylase domains in human SIRT1-7, color coded to match the phylogenetic class from (A): green, Class I; blue, Class II; red, Class III; yellow, Class IV. Reused with permission from (Hirschey, 2011).
1.1 Enzymatic Activity of the Mitochondrial Sirtuins

The sirtuins were initially described as deacetylases that target only acetyl-lysine for removal, but the enzymatic activities of this family of proteins is broader than originally realized. Sirtuins are now appreciated to target several acyl-lysine modifications for removal, including malonyl- (Du et al., 2011; Peng et al., 2011), succinyl- (Du et al., 2011), and glutaryl-lysine (Tan et al., 2014) modifications, as well as long-chain acyl-modifications (Feldman et al., 2013). Lysine acetylation is thought to occur non-enzymatically (Ghanta et al., 2013; Wagner and Hirschey, 2014; Wagner and Payne, 2011), which increases under conditions of elevated acetyl-CoA levels (Wagner and Hirschey, 2014). Acylation, referring to a wide range of modifications, of mitochondrial proteins is generally repressive (Chhoy et al.), though lysine acylation has been described to activate the enzyme activity in a few cases (Park et al., 2013).

SIRT3 is a lysine deacetylase that controls several pathways, including lipid metabolism and oxidative stress (Hirschey et al., 2010). The enzymatic activity of SIRT4 has been described as an ADP-ribosyltransferase (Haigis et al., 2006), a deacetylase (Michishita et al., 2005), a lipoamidase (Mathias et al., 2014), and a deacylase (removes methylglutaryl, hydroxymethylglutaryl, and 3-methylglutaconyl groups from lysine residues) (Anderson et al., 2017), which appears to be the primary activity of SIRT4. SIRT4 has been shown to influence the metabolism of amino acids (Anderson et al., 2017), lipids, and the TCA cycle (Haigis et al., 2006; Jeong et al., 2013). SIRT5 is a lysine
demalonylase, desuccinylase, and deglutarylase which controls several metabolic pathways, such as the urea cycle (Tan et al., 2014).

1.1.1 Proteomic characterization of acyl-lysine modifications

While the enzymatic activities of SIRT3 and SIRT5 are well characterized, the breadth and depth of acylation remains a nascent field of investigation. In order to being to answer this question, a number of proteomic surveys have been conducted to determine the landscape of acetylation, succinylation, malonylation, and glutarylation. Using SIRT3-deficient mitochondria extracted from mouse liver mitochondria, Lombard et al. showed that acetylation accumulates on mitochondrial proteins in the absence of SIRT3 (Lombard et al., 2007). Using an unbiased approach, they were able to identify glutamate dehydrogenase (GDH) as an acetylated protein regulated by SIRT3. Since these initial discoveries of SIRT3 substrates, more sensitive and high throughput proteomic approaches to identifying acylated proteins have been developed. In 2009, Choudhary et al. identified 3600 lysine acetylation sites on 1750 proteins involved in cellular processes including DNA damage and repair, chromatin remodeling, and cell cycle (Choudhary et al., 2009). This study showed the breadth of lysine acetylation. More recently, acyl proteomics performed on SIRT3 and SIRT5 deficient tissues compared to wildtype tissues has provided insight as to what proteins and pathways are potentially regulated by sirtuin mediated deacylation.
In a study of SIRT3KO and WT mouse livers, Rardin et al. identified 2,187 sites of lysine acetylation. Interestingly, about 14% and 17.5% of these sites were unique to WT and SIRT3KO livers, respectively, suggesting that the landscape of acetylation changes. By analyzing proteins with 2-fold or higher changes in acetylation in the absence of SIRT3, the authors of this study identified a number of metabolic pathways that are likely regulated by SIRT3. The top pathways include fatty acid oxidation, the TCA cycle, branched chain amino acid catabolism, ketone body metabolism, and the electron transport chain (Rardin et al., 2013b). Similar approaches have identified SIRT3 as a potential regulator of metabolism and have suggested that regulation may be tissue specific (Dittenhafer-Reed et al., 2015).

SIRT5 removes succinyl, malonyl, and glutaryl groups. Addition of succinyl, malonyl, and glutaryl groups changes the charge of the modified lysine from a positive to negative change. The unique acyl binding pocket of SIRT5 coordinates the interaction of carboxylates with this enzyme in order for the deacylation reaction to occur (Du et al., 2011). The role of SIRT5 mediated desuccinylation is currently the most well explored of these three post translational modifications. Park et al. conducted a comprehensive survey of the succinylome in mouse liver tissue and discovered 2,565 succinylation sites on 779 proteins, indicating that the number of succinylated proteins is comparable to the number of acetylated proteins. Similar to acetylation, succinylation in SIRT5KO tissue occurs on a statistically significant number of proteins in metabolic pathways including
branched chain amino acid degradation, the TCA cycle, and fatty acid oxidation (Park et al., 2013). Complementing the succinylome survey by Park et al., Rardin et al. completed a succinylome survey in SIRT5KO and WT mouse livers and identified 1,576 sites of lysine succinylation on 392 proteins. Pathway analysis of SIRT5 targets (two-fold higher succinylation in the SIRT5KO liver) indicated that SIRT5 likely regulates multiple metabolic pathways including fatty acid oxidation, branched chain amino acid catabolism, the TCA cycle, ATP synthesis, and ketone body synthesis (Rardin et al., 2013a). Interestingly, investigation of the malonylome identified a 1,137 sites of lysine malonylation on 430 proteins with many sites occurring on cytoplasmic proteins (Nishida et al., 2015). In this study, it was determined that glycolysis was the main pathway regulated by SIRT5 mediated demalonylation (Nishida et al., 2015). Characterization of the glutarylome reported fewer sites of lysine glutarylation (683 lysines in 191 proteins), suggesting that this modification is not abundant or development of a more sensitive pan-glutaryl-lysine antibody is necessary to fully understand the glutarylation landscape (Tan et al., 2014). Most studies analyzing various acylomes have been conducted in liver tissue. Given the potential tissue-specific effects of sirtuins, it will be important to determine targets of sirtuins in different tissues.

Of the mitochondrial sirtuins, synthesizing targets of SIRT3 and physiology has been the most-well investigated (for a recent review see (van de Ven et al., 2017)). The lack of a complete understanding of SIRT4 enzymatic activity impedes efforts to
understand the physiologic role of SIRT4. Despite a thorough investigation of the acylation landscape and understanding of SIRT5 enzymatic activity, little is known about the physiological role of SIRT5. We and others (Nishida et al., 2015) have shown that SIRT5 protein is high in the heart (Figure 2 A and C) and that succinylation increases dramatically in SIRT5KO compared to WT hearts (Figure 2 A and C). These data suggest that SIRT5 mediated desuccinylation may be important in cardiac function and metabolism. Therefore, we focus on the current understanding of the cardiac roles of the mitochondrial sirtuins.
Figure 2: Tissue panel in SIRT5KO mouse model

(A) Western blot showing succinylation and SIRT5 expression in tissues isolated from WT and SIRT5KO male mice. Representative of 3 independent experiments. (B) Quantification of succinylation fold change (KO/WT) in each tissue, signal normalized to total protein. (C) Quantification of SIRT5 in each WT tissue. Normalized to total protein and then to SIRT5 expression in the WT heart. Quantification is expressed as mean ± SEM of three independent experiments.

1.2 Physiological Roles of the Mitochondrial Sirtuins in the Heart

1.2.1 SIRT3 is cardioprotective

The role for SIRT3 in maintaining normal cardiac physiology is well supported, as is a role for its reduction in pathophysiological states. Ablating SIRT3 in the heart results in hyperacetylation of metabolic enzymes, hypertrophy, and markedly reduced
 (>50%) ATP levels, demonstrating an important role for SIRT3 in regulating cardiac bioenergetics (Ahn et al., 2008; Sundaresan et al., 2009). SIRT3KO mice develop spontaneous cardiac hypertrophy with aging (Hafner et al., 2010) and have increased susceptibility to several pharmacological-induced or other cardiac stresses (Pillai et al., 2010; Porter et al., 2014). Consistent with this idea, several studies report mitochondrial protein hyperacetylation of cardiac proteins in both mouse models of heart failure and failing human hearts (Horton et al., 2016).

Indeed, many studies have concluded that hyperacetylation of mitochondrial proteins is a driver of metabolic dysfunction in the heart (Alrob et al., 2014; Hirschey et al., 2010; Karamanlidis et al., 2013; Lee et al., 2016). For example, hyperacetylation of cardiac LCAD and other enzymes involved in fatty acid oxidation (FAO) increased their activities in high-fat diet feeding in mice (i.e. a model of obesity-induced cardiomyopathy) and in a Sirt3−/− (SIRT3KO) mouse (Alrob et al., 2014). The authors conclude that the higher rates of FAO likely reduced cardiac efficiency in this model of obesity, and would likely reduce the cardiac efficiency of stressed SIRT3KO mice. In contrast to this work, an earlier study showed protein hyperacetylation was associated with reduced FAO rates in the hearts of fasted animals (Hirschey et al., 2010). This discrepancy suggests that the type of stress may be important for different effects of acetylation on proteins. Future studies elucidating this context-specific regulation via
acetylation of target proteins will be important for understanding the role of acetylation in a given cardiac pathology.

1.2.2 SIRT4 may be detrimental to cardiac function

Like the other mitochondrial sirtuins, SIRT4 is highly expressed in tissues with high metabolic demand, including heart, kidney, liver, and brain (Haigis et al., 2006). While less is known about SIRT4, it might be detrimental to cardiac function. Ang II-induced cardiac hypertrophy and fibrosis were reduced in a mouse model of SIRT4 deficiency. (Luo et al., 2016). The authors of this study conclude that SIRT4 prevents the binding of SIRT3 to SOD2, a critical protein for minimizing reactive oxygen species (ROS) in the heart. Decreased binding of SIRT3 to SOD2 enabled increased acetylation and reduced activity of SOD2, which augmented oxidative stress (Luo et al., 2016). Additionally, early studies with a mouse model of cardiomyocyte-specific overexpressing SIRT4 show more pronounced cardiac dysfunction and dilation after twelve weeks of pressure overload induced cardiac hypertrophy (Koentges et al., 2017). Clearly, additional studies are required to understand the role of SIRT4 in cardiac physiology and pathophysiology.

1.2.3 Loss of SIRT5 is detrimental to cardiac function under stress

Compared to the known cardioprotective role of SIRT3 in the heart, the role of SIRT5 in maintaining normal cardiac function has only recently been studied. In an early study describing the metabolic characterization of Sirt5−/− (SIRT5KO) mice, no overt
cardiac differences were found, suggesting that SIRT5 is metabolically unremarkable (Yu et al., 2013). Specifically, heart weight, heart rate, and systolic blood pressure of SIRT5KO animals undergoing the stress of a chronic high-fat diet remained unchanged compared to WT controls. However, a stress is often required to elicit a phenotype in sirtuin-deficient animals; indeed, a recent study showed cardiac stress is needed to study the role of SIRT5 in heart (Boylston et al., 2015). While SIRT5 targets multiple protein modifications, protein succinylation is uniquely elevated in SIRT5KO hearts (Nishida et al., 2015), suggesting that reversible succinylation of metabolic proteins may be an unappreciated way in which cardiac metabolism is regulated. A proteomic survey of the mouse heart succinylome found that, similar to studies in the liver, a large number of metabolic pathways are significantly succinylated in the absence of SIRT5, including fatty acid metabolism, branched-chain amino acid catabolism, TCA cycle, oxidative phosphorylation, stress pathways, ketogenesis, and pyruvate metabolism (Boylston et al., 2015).

In a model of ischemia-reperfusion, SIRT5KO hearts had a greater infarct area post-reperfusion compared to WT controls, as well as slightly elevated ROS (Boylston et al., 2015). Succinylation activated succinate dehydrogenase (SDH) in the SIRT5KO heart, and inhibition of SDH normalized infarct size to wild-type levels (Boylston et al., 2015). Together, these data suggest that SIRT5 influences cardiac stress response by decreasing SDH activity via desuccinylation. In addition to ischemia-reperfusion, aging is another
stress that elicits a phenotype in SIRT5KO mouse hearts (Sadhukhan et al., 2016). At 39 weeks, the hearts of SIRT5KO mice were significantly hypertrophied and had increased fibrosis compared to controls. Additionally, echocardiograms show that both shortening fraction and ejection fraction in SIRT5KO hearts are decreased at 8 weeks and 39 weeks of age compared to WT controls (Sadhukhan et al., 2016). Mechanistically, succinylation and subsequent inhibition of the mitochondrial trifunctional protein alpha subunit was a key enzyme influencing the observed cardiomyopathy in the aging SIRT5KO mouse heart.

1.2.4 Mitochondrial sirtuins require a stress to elicit a phenotype

The work described above provides an overview of the physiological roles of the mitochondrial sirtuins in the heart. Remarkably, genetic mouse models of SIRT3 ablation, SIRT4 ablation, or SIRT5 ablation show few cardiac phenotypes at baseline. While SIRT3KO mice develop spontaneous cardiac hypertrophy (Hafner et al., 2010; Martin et al., 2017), it does not appear to be detrimental to overall lifespan. Indeed, there appear to be no overt phenotypes in any of the mitochondrial sirtuin mouse knock-out models under basal conditions. This is somewhat surprising given the dramatic increase in acyl-lysine modifications regulated by the sirtuins—hyperacetylation in SIRT3KO mice (Hirschey et al., 2010); and hypermalonylation (Nishida et al., 2015), hypersuccinylation (Nishida et al., 2015; Rardin et al., 2013a; Yu et al., 2013), and hyperglutarylation (Tan et al., 2014) in SIRT5KO mice. Instead, it appears that
hyperacylation may contribute to the susceptibility of disease states and diminished healthspan. For example, loss of SIRT3 has been shown to accelerate the development of metabolic syndrome in a mouse model of high fat diet feeding (Hirschey et al., 2011). Additionally, SIRT5 ablation may lead to reduced flux through fatty acid oxidation (Rardin et al., 2013a; Sadhukhan et al., 2016) and ketogenesis (Rardin et al., 2013a).

It has recently been hypothesized that mitochondrial sirtuins exist to remove acyl-lysine modifications as a way to deal with carbon stress that comes from reactive acyl-CoA species (Wagner and Hirschey, 2014). In this model, reactive acyl-CoA species are produced from the oxidation of fatty acids, glucose, and amino acids. The fate of the reactive acyl-CoA species may be nonenzymatic lysine acylation that reduces enzyme function. It has been proposed that enzyme function could be inhibited by acylation due to a change in enzyme structure, or by affecting substrate or cofactor binding (Chhoy et al., 2016). Sirtuins act to remove acyl-lysine modifications to restore enzymatic activity and maintain metabolic homeostasis (Figure 3). The addition of a metabolic stress, such as high fat diet feeding, may overwhelm the system with an abundance of acyl-CoA species—resulting in hyperacylation and decreased enzyme function. It remains to be understood if there are specific sites of lysine acylation that contribute to decreased enzyme function, or if it is the acyl-lysine landscape that ultimately leads to increased susceptibility to disease (van de Ven et al., 2017).
(A) Reactive acyl-CoA metabolites can nonenzymatically react with nucleophilic protein residues in order to form posttranslational modifications that can compromise protein function and that could be considered carbon stress. (B) Nonenzymatic acyl modifications can be targeted for removal by the sirtuin family of NAD+-dependent lysine deacylases in order to restore protein function and cellular health, which is part of a global protein quality-control system. Adapted and reused with permission from (Wagner and Hirschey, 2014).

1.3 Cardiac Structure, Function, and Metabolism in LV Hypertrophy

The stresses of ischemia-reperfusion and aging have shown that depletion of SIRT5 under these conditions is detrimental to cardiac function. In this dissertation, the stress of pressure overload is used to induce cardiac hypertrophy. A number of
structural, functional, and metabolic changes occur during the development of left ventricle hypertrophy which will be discussed here.

1.3.1 Cardiac structural and functional changes with pressure overload induced hypertrophy

Left ventricle cardiac hypertrophy is defined as an enlargement of cardiomyocyte cells and can occur due to a number of stresses on the heart including conditions that cause pressure overload (i.e. hypertension or aortic valve stenosis) or volume overload (i.e. chronic exercise or pregnancy). Growth of cardiomyocytes occurs though an increase in protein production and the resulting hypertrophy decreases ventricular wall stress by increasing wall thickness (Maillet et al., 2013).

With increased cardiomyocyte size, the morphology of the left ventricle changes. Two types of hypertrophy are frequently adapted; eccentric hypertrophy or concentric hypertrophy. With eccentric hypertrophy, cardiomyocytes grow in both length and width, resulting in an increase in left ventricle volume and a coordinated increase in septal wall and left ventricle wall thickness (Maillet et al., 2013). This usually occurs in response to non-pathological stresses and may improve cardiac function. In contrast, concentric hypertrophy usually develops in response to pathological stresses and is maladaptive. Concentric hypertrophy is characterized by cardiomyocyte increases in length greater than width and results in a reduced left ventricle volume and increases in septum and left ventricular wall thickness (Maillet et al., 2013). A hypertrophic response to pathological stimuli ultimately proceeds to dilation because the heart cannot
overcome the chronic pressure load. The walls of the septum and left ventricle become thin, left ventricular volume increases, and ejection fraction decreases (Figure 4).

Additional morphological changes that occur with pathological hypertrophy include increased fibrosis which contribute to heart stiffness (Maillet et al., 2013) and an increase in the proportion of myosin heavy chain-beta (MHC-β) to MHC-α, which influences the efficiency of the heart (Taegtmeyer et al., 2010a). In concert with this mechanical remodeling of the ventricle, the heart become energy starved and eventually fails.

Pathologic stimuli that result in pressure overload such as hypertension and aortic valve stenosis cause left ventricle hypertrophy. This hypertrophy is characterized by a thickening of the septum wall and left ventricle wall that results in a decrease in left ventricle volume. Fibrosis and cardiac dysfunction may be present in this stage. Ultimately, chronic stress leads to further morphological changes to the left ventricle that leads to cardiac dilation. In this stage, left ventricle volume increases at the expense of decreased cardiac function due to thin septum and left ventricle walls. Adapted and reused with permission from (Berry et al., 2007).

**Figure 4: Progression of left ventricle hypertrophy to cardiac dilation**
1.3.2 Changes in cardiac metabolism with pressure overload induced hypertrophy

The adult heart is considered an “omnivore” and metabolically flexible, in that it can use many energy substrates including fatty acids, glucose, lactate, pyruvate, ketones, and amino acids to generate ATP to meet energy demands (Masoud et al., 2013). The heart requires an enormous amount of ATP to maintain constant pumping to deliver oxygenated blood throughout the body. Although metabolically flexible, the preferred substrates of the heart are fatty acids and glucose. Under normal conditions, 60%-90% of ATP generated comes from oxidation of fatty acids, and 10%-40% of ATP is generated from glucose oxidation (Sankaralingam and Lopaschuk, 2015). Reserve energy is stored in the heart as phosphocreatine (PCr), and phosphoryl transfer to ADP via creatine kinase generates ATP approximately 10 times faster than ATP synthesis in the mitochondria and is thus important to meet demands of the heart during increased workloads (Ingwall, 2009).

Many stimuli including pressure overload, ischemia, and diabetes can lead to altered metabolic fluxes in the heart (Taegtmeyer et al., 2010b). These shifts in metabolism include a decrease in fatty acid and glucose oxidation, and an increased reliance on glycolysis (Figure 5) (Lopaschuk, 2017). Shifts in cardiac metabolism with pathological hypertrophy appear to be driven by transcriptional changes, with downregulation of enzymes in fatty acid oxidation, an increase in glucose uptake by upregulation of GLUT1, and increase in inhibition of PDH by PDK-mediated
phosphorylation (Sankaralingam and Lopaschuk, 2015). Importantly, an uncoupling of glycolysis from glucose oxidation is detrimental to cardiac efficiency (Masoud et al., 2013) and it has been suggested that defects in oxidative metabolism precede the onset of cardiac dysfunction (Sankaralingam and Lopaschuk, 2015). Indeed, it has been shown that NAD$^+$ levels decline with cardiac hypertrophy (Katsyuba and Auwerx, 2017), which can contribute to a reduction in mitochondrial function (Braidy et al., 2011) and disease pathogenesis (Forbes, 2016; Mericskay, 2015). Thus, elevating intracellular NAD$^+$ levels has become of interest as a therapeutic means to treat the metabolic dysfunction that occurs with heart failure.
Figure 5: Alterations in myocardial energy substrate metabolism in heart failure

Under normal conditions, the heart uses primarily fatty acids and glucose to produce ATP via oxidation. Under conditions of hypertrophy, the heart switches to depending more on glycolysis to produce ATP. Decreased in fatty acid oxidation are due to decreases in enzymes of fatty acid oxidation including CD36, CPT1, and LCAD. Glucose oxidation is impaired due to a decrease in GLUT4 expression, and an increase in PDK-mediated inhibitory phosphorylation of PDH. Glycolysis is enhanced by increased expression of GLUT1. Ultimately, these shifts in metabolism are insufficient to continue to produce enough ATP to maintain cardiac function and the heart undergoes energetic failure.
1.4 Therapeutic Potential of Sirtuin Activation with NAD⁺

1.4.1 NAD⁺ synthesis and cellular function

In mammals, NAD⁺ is synthesized by de novo synthesis as well as salvage from dietary niacin or vitamin B₃ compounds. De novo synthesis originates from the amino acid tryptophan, which is metabolized to form biosynthetic intermediates. These intermediates ultimately generate the pyridine moiety of NAD⁺ (i.e. nicotinamide) and then form NAD⁺. Salvage of precursors including nicotinic acid (NA), nicotinamide (NAM), and nicotinamide riboside (NR) are taken up from the diet and recycled to generate cellular NAD⁺ (Cantó et al., 2015; Sauve, 2007). In the heart, nearly all NAD⁺ is generated from salvage of NA, NAM and NR—though de novo synthesis can occur under some conditions (Mori et al., 2014).

NAD⁺ carries out several important cellular functions (Cantó et al., 2015). NAD⁺ is a hydride acceptor, which forms the reduced dinucleotide NADH and is vital for driving reduction-oxidation (redox) reactions in energy production. Additionally, NAD⁺ is a precursor for the phosphorylated dinucleotides NADP⁺ and NADPH, which are required for several cellular biosynthetic pathways and to protect cells from reactive oxygen species (ROS). Finally, NAD⁺ acts as an enzyme substrate for several non-redox reactions, such as signaling pathways, where its adenine diphosphate ribose (ADPR) moiety is transferred to proteins or serves as a signaling molecule to regulate cellular function (e.g. sirtuins, ADP-ribosyltransferases, and ADPR cyclases).
1.4.2 Effect of NAD$^+$ on sirtuin function

The dependence of sirtuins on NAD$^+$ links energy metabolism and sirtuin function (Imai et al., 2000). Given the depletion of NAD$^+$ in with aging and disease, it has been hypothesized that one way to boost sirtuin activity is through NAD$^+$ supplementation. Early work of the effect of NAD$^+$ on sirtuin function in non-mammalian systems has provided the basis for further investigation of the therapeutic effects of boosting sirtuin activity with NAD$^+$ supplementation. Yeast sirtuins can be activated by manipulating NAD$^+$ biosynthetic pathways or increasing concentrations of NAD$^+$. For example, increasing Npt1 (nicotinate phosphoribosyltransferase, an enzyme in NAD$^+$ biosynthesis), increased yeast sirtuin (Sir2) activity and extended yeast life span by up to 60% without changing NAD$^+$ levels (Anderson et al., 2002). Similar effects in lifespan extension were observed with NR supplementation, which increased in NAD$^+$ levels (Belenky et al., 2007). However, supplementation of NA in yeast had no effect on Sir2 activity (Bitterman et al., 2002), demonstrating that different NAD$^+$ precursors elicit different biological effects, perhaps due to differences in uptake, availability, or fate.

Recently, investigation into NAD$^+$ supplementation and the effects on sirtuin activity in higher organisms have yielded similar results. Exogenous NR or NAM increases levels of NAD$^+$, improved mitochondrial function, and increased lifespan in C. elegans (Mouchiroud et al., 2013). In a mouse model of high-fat diet-induced type 2 diabetes, supplementing NMN restored NAD$^+$ levels, which was partially attributed to
SIRT1 activation (Yoshino et al., 2011). Based on these studies, and others, supplementation with NAD$^+$ precursors or boosting activity of enzymes in the NAD$^+$ salvage pathway is being explored as one strategy of sirtuin activation to treat diseases. In particular SIRT1, the mammalian sirtuin homolog of yeast Sir2, has been extensively studied for its influence on aging and lifespan, and its role in mediating effects of NAD$^+$ supplementation (Giblin et al., 2014; Imai and Guarente, 2014).

1.4.3 Boosting SIRT3 activity is cardioprotective

NAD$^+$ supplementation studies have identified a role for SIRT3 in mediating some of the beneficial effects of NAD$^+$ therapy in the heart. Early studies showed that treatment of mice or mouse embryonic fibroblasts (MEFs) with NR increased NAD$^+$ levels and activated SIRT1 and SIRT3 (Canto et al., 2012). NR supplementation also reduced acetylation of specific targets of SIRT3, including superoxide dismutase 2 (SOD2) and NADH:Ubiquinone oxidoreductase subunit A9 (NDUFA9), suggesting that SIRT3 is activated by NR treatment. Additionally, SIRT3KO mice are more sensitive to isoproterenol (ISO)-induced cardiac hypertrophy (Pillai et al., 2010) and treatment with exogenous NAD$^+$ blocks ISO-induced hypertrophy in a SIRT3-dependent manner. Finally, NMN supplementation studies demonstrate reduced acetylation of cardiac mitochondrial proteins in a mouse model of complex I deficiency (Karamanlidis et al., 2013; Lee et al., 2016), potentially via activation of SIRT3.
Together, these data show that NAD$^+$ has the capacity to activate SIRT3 in the heart and improve cardiac function. So far, NMN and NAD$^+$ have been shown to modulate acetylation (Karamanlidis et al., 2013) and activate SIRT3 (Pillai et al., 2010), respectively. Overall, these studies suggest that NMN is an effective means to activate cardiac SIRT3, in order to off-set functional cardiac decline during heart failure. Similar to SIRT3, there is evidence that SIRT5 may be cardioprotective. To date, there are no studies exploring the effect of NAD$^+$ boosting strategies on SIRT5.

1.5 Project Goals

Given the evidence thus far that SIRT5 may have a role in responding to cardiac stress and that succinylation is abundant in the SIRT5KO heart, it is important to understand the role of SIRT5 mediated desuccinylation in the heart. Since it appears that there are no defects in cardiac function in SIRT5KO mice under basal conditions, I hypothesized that a stress would be required to determine a protective effect of SIRT5 in the heart. Additionally, I hypothesized that multiple sites of lysine succinylation would contribute to the overall phenotype observed.

To address this hypothesis, genetic mouse models were exposed to a well characterized model of pressure overload induced cardiac hypertrophy—transverse aortic constriction (TAC). Two main mouse models were used: 1) a whole body SIRT5KO mouse and 2) a cardiomyocyte tamoxifen-inducible heart specific SIRT5KO
mouse. In order to characterize cardiac structure and function following TAC, the methods of serial echocardiogram and pressure volume loops with inferior vena cava suppression were used. TAC surgery and subsequent morphological and functional analyses were performed in collaboration with Dennis Abraham, PhD, and Lan Mao, MD of the Duke Cardiovascular Physiology Core. Mechanistic studies included metabolomic (in collaboration with Jason Locasale, PhD, Duke University) and succinyl-proteomic (in collaboration with Cell Signaling Technologies, Inc. and Paul Grimsrud, PhD, Duke University) profiling of heart samples. Additionally, Western Blot and RT-qPCR were used to further gain mechanistic insight.

Chapter 3 of this dissertation characterizes the response of the whole body SIRT5KO mouse to pressure overload induced hypertrophy compared to littermate controls. A combination of succinyl-proteomics and metabolomics investigates the effect of TAC on cardiac metabolism in SIRT5KO mice. To specifically investigate the role of SIRT5 in cardiomyocytes, a heart-specific, inducible SIRT5KO mouse is generated and exposed pressure overload via TAC surgery. The results of this investigation are presented in Chapter 4. Additionally, a novel observation of the time course of succinylation in the heart specific SIRT5KO mouse model is investigated using succinyl-proteomics. Collectively, the results of these studies provide new insight into the role of SIRT5 mediated desuccinylation in regulating cardiac function and metabolism.
With their activity coupled to NAD$^+$, the mitochondrial sirtuins offer a potential therapeutic target of diseases associated with NAD$^+$ depletion, such as aging and heart failure. Previous studies have suggested that activating SIRT3 with NAD$^+$ supplementation strategies is beneficial to cardiac function. It will be of interest to researchers to investigate the effect of NAD$^+$ boosting strategies on SIRT5. Thus far, this work has been hindered because, even though the enzymatic activity of SIRT5 is well characterized, the understanding of the physiological role of SIRT5 is not well understood. Determining the physiological roles of SIRT5 will enable the field to make advances in harnessing the therapeutic potential of SIRT5. Further, this study greatly increases the known number of sites of protein lysine succinylation in the heart. This database provides a resource to further define the regulation of many proteins involved in a variety of mechanisms including cardiac contraction and metabolism. Finally, this study makes an important contribution to further defining the mechanism of protein lysine succinylation.

This study thoroughly characterizes cardiac function and morphology in response to TAC when Sirt5 is ablated in the germline and in a cardiomyocyte-specific manner. However, proteomic and metabolomic studies do not provide a definitive mechanism of the phenotypes observed in the whole body SIRT5KO mouse with TAC. This investigation lays the groundwork for further exploring the cellular mechanisms described here, and posits potential mechanisms to test based on synthesis of the data.
generated here and in other studies. Conclusions and next steps are fully discussed in Chapter 5 of this dissertation.
2. Materials and Methods

2.1 Animals

2.1.1 Whole body SIRT5KO

SIRT5KO mice were obtained from Jackson Laboratory (Bar Harbor, ME, stock #012757) and backcrossed for 10 generations onto a C57BL/6J background obtained from Jackson Laboratory (Bar Harbor, ME, stock #000664). Mice backcrossed for 7-10 generations were used in these studies. Mice used for in cardiac function studies had a mixed Nnt background (see Appendix A for a discussion on the effect of Nnt on cardiac function) while mice used for mechanistic studies (proteomics, metabolomics, Westerns, and RNA) had a Nnt<sup>Mat/Mat</sup> background. Mice were group-housed on a 12-hour light/dark cycle with free access to water and PicoLab Rodent Diet 20 (LabDiet #5053, St. Louis, MO). Male and female breeder genotypes were SIRT5<sup>+/−</sup> in order to obtain litters with SIRT5KO and WT littermate controls. Age, sex, genotype, and number of animals used per study are provided in the corresponding figure legends. All <i>in vivo</i> procedures were performed on healthy animals in accordance with the Duke Institutional Animal Care and Use Program.

2.1.2 Heart specific SIRT5KO mouse model

To generate the tamoxifen-inducible cardiomyocyte-specific SIRT5KO mouse, we crossed SIRT5<sup>fl/fl</sup> females (Yu et al., 2013) (generous gift from Johanne Auwerx, EPFL, Lausanne, Switzerland) with αMHC-MerCreMer<sup>+/−</sup> (obtained from Jackson Laboratory,
Bar Harbor, ME; stock #005657) males to generate littermates with the following genotypes: SIRT5<sup>fl/fl</sup>; αMHC-MerCreMer<sup>−/−</sup> (hereafter referred to as fl/fl) and SIRT5<sup>fl/fl</sup>; αMHC-MerCreMer<sup>+/−</sup> (hereafter referred to as fl/fl;MCM). Additionally, the floxed alleles were crossed out of this line by crossing SIRT5<sup>fl/fl</sup>; αMHC-MerCreMer<sup>−/−</sup> males with C57BL/6J females from Jackson Laboratory (Bar Harbor, ME, stock #000664) to generate a line of non-floxed αMHC-MerCreMer<sup>−/−</sup> and αMHC-MerCreMer<sup>+/−</sup> mice to generate αMHC-MerCreMer<sup>−/−</sup> (hereafter referred to as MCM) mice to control for Cre toxicity. All of these mice are on the C57BL/6J background. Mice were group-housed on a 12-hour light/dark cycle with free access to water and PicoLab Rodent Diet 20 (LabDiet #5053, St. Louis, MO). To induce MerCreMer expression, tamoxifen citrate diet (Envigo Tekland Diets, Madison, WI; TD.130860) was fed for 8-10 days. Tamoxifen citrate pellets were moistened to encourage eating and regular chow was fed over the weekends (after 3-5 days of tamoxifen citrate feeding) to decrease weight loss with new diet. Age, sex, genotype, and number of animals used per study are provided in the corresponding figure legends. All in vivo procedures were performed on healthy animals in accordance with the Duke Institutional Animal Care and Use Program.

2.1.3 Genotyping of mouse models

To determine genotypes, ear tissue was clipped and DNA was extracted using Chelex 100 resin (Bio-Rad, Hercules, CA; 1421253). Tissue was incubated in 250 μl 112.5 mg/mL Chelex 100, 0.1 mg/mL proteinase K, and 0.1% Tween-20 for 45 minutes at 55 °C
followed by 15 minutes at 95 °C. Samples were gently mixed by flicking and centrifuged for 1 minute at 10,000 x g. Supernatant was used as a template for PCR reactions. Sirt5 genotype was determined using forward primer one with the sequence CCA CGG AAC CTT ATT TAA AAC TCG, forward primer two with the sequence AGG AGG TGG CAA AGG TCT TGC, and a common reverse primer with the sequence GAT TCT GGC GTC TTG AGT CTC. The Nnt genotype was determined using primer sequences from Jackson lab. Three primers are used in this genotyping reaction as follows: p7332 with sequence GTA GGG CCA ACT GTT TCT GCA TGA, p7333 with sequence GGG CAT AGG AAG CAA ATA CCA ACT TG, and p7334 with sequence GTG GAA TTC CGC TGA GAG AAC TCT T. In order to determine if Sirt5 alleles were floxed, a forward primer with the sequence TGT GCT TGT ACG TGC TGT GC and a reverse primer with the sequence CCC CTC ACT CAG CTC ACA AA were used in the PCR reaction. Insertion of the MHC-MerCreMer transgene was confirmed using a forward primer with sequence ATA CCG GAG ATC ATG CAA GC and a reverse primer with sequence AGG TGG ACC TGA TCA TGG AG. These reactions were concurrently run with an internal positive control for interleukin-2, a gene present in every mouse, using forward primer with sequence CTA GGC CAC AGA ATT GAA AGA TCT and a reverse primer with sequence GTA GGT GGA AAT TCT AGC ATC ATC C. All genotyping PCR reactions were performed with Phire Hot Start II DNA Polymerase (ThermoFisher Scientific).
2.2 Western Blots

Left ventricle tissue (whole or part) was dissected and flash frozen. Tissue was homogenized in RIPA buffer with protease inhibitors using a rotor. A Teflon pestle rotated at 1000 rpm was used to homogenize tissues with approximately 10 strokes. Tissue was spun down for 10 minutes at 10,000 x g at 4 °C. The supernatant was collected and a BCA assay (Sigma) was run to determine protein concentration. Protein concentrations for each sample were normalized to 2 μg/μl in 4X Laemmli Sample Buffer (Bio-Rad). Whole-cell protein extracts were resolved by SDS-PAGE using stain free Bio-Rad gels and transferred to nitrocellulose membranes using Bio-Rad’s Trans-Blot Turbo. Total protein was quantified with the Gel Doc XR+ (Bio-Rad) using stain free technology. The membranes were blocked in 5% milk in TBS-T (TBS containing 0.1% Tween 20) for 1 hour at room temperature and probed with primary antibodies in TBS-T. For anti-acyl-K blots, 3% BSA was added to the primary antibody solution. After incubation with infrared dye-conjugated antibodies, the blots were developed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Commercial antibodies were used as follows: anti-PDH E1α1 subunit from Cell Signaling (32055), anti-Phospho-PDH from Calbiochem (AP1062), anti-Succinyl-K from PTM (401), anti-Malonyl-K from Cell Signaling (14942), anti-Acetyl-K from Cell Signaling (9441), and anti-Glutaryl-K from Cell Signaling (generous gift), anti-Acetyl CoA Carboxylase from Cell Signaling (3662), anti-Phospho-Acetyl CoA Carboxylase
(Ser79) from Cell Signaling (3661), anti-AMPKα from Cell Signaling (2603), anti-Phospho-AMPKα (Thr172) from Cell Signaling (2535), anti-total OXPHOS cocktail from MitoSciences (ab110413), anti-4EBP1 from Cell Signaling (9644S), anti-Phospho-4EBP1 from Cell Signaling (2855S), anti-CaMKII-alpha from Cell Signaling (D10C11), and anti-Phospho-CaMKII-alpha from Cell Signaling (D21E4). Anti-SIRT5 was provided as a generous gift from Leonard Guarente (MIT, Cambridge, MA).

### 2.3 RT-qPCR

Animals were euthanized by exposure to CO₂ for 5 minutes and immediate excision of the whole heart. Heart was washed in PBS and the left ventricle was dissected. A small (approximately 50 mg) piece of the left ventricle apex was flash frozen immediately for downstream RNA analysis. RNA was extracted from tissues using RNeasy Mini Kit from Qiagen (74106). cDNA was made from 750 ng RNA using iScript cDNA Synthesis Kit from Bio-Rad (170-8890) and diluted 1:8 with nuclease free water. Amplification was performed using iTaq Universal SYBR Green Supermix from Bio-RAD (1725121) on the QuantStudio 6 Flex (ThermoFisher Scientific). RT-qPCR reaction mix contained 2.5 µl 1:8 cDNA, 0.5 µl of 10 µM forward and reverse primer mix, 4 µl SYBR, and 1 µl nuclease free water. 36B4 was used as a reference gene and relative expression was calculated using the △△CT method. Forward and reverse primers for genes of interest are listed in Table 1.
Table 1: Sequences of primers used in RT-qPCR

Forward and reverse primer sequences for ribosomal protein, large, P0 (36B4); acyl-Coenzyme A dehydrogenase, long chain (Acadl); acyl-Coenzyme A dehydrogenase, medium chain (Acadm); acyl-Coenzyme A dehydrogenase, very long chain (Acadvl); adrenergic receptor, beta 1 (Adrb1); adrenergic receptor, beta 2 (Adrb2); natriuretic peptide type A (Anf); natriuretic peptide type B (Bnp); CD36 molecule (Cd36); collagen, type I, alpha 1 (Col1a1); collagen, type III, alpha 1 (Col3a1); carnitine palmitoyltransferase 1b, muscle (Cpt1b); solute carrier family 2 (facilitated glucose transporter), member 1 (Glut1); solute carrier family 2 (facilitated glucose transporter), member 4 (Glut4); interleukin 6 (Il6); interleukin 10 (Il10); lactate dehydrogenase A (Ldha); pyruvate dehydrogenase A (Pdk4); peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (Pgc1a); peroxisome proliferator activated receptor alpha (Ppara); peroxisome proliferator activator receptor delta (Ppard); regulator of calcineurin 1 (Rcan1.4); and sirtuin 5 (Sirt5).

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<td>5'-GCCATTTCCTCCGACTTTT-3'</td>
</tr>
<tr>
<td>Pgc1a</td>
<td>5'-GACTGCTGACAGGAGAGC-3'</td>
<td>5'-GGGTATGATGCTGCTGAG-3'</td>
</tr>
<tr>
<td>Ppara</td>
<td>5'-AACATGGGAGCAGTGTGTTT-3'</td>
<td>5'-AACATGGGAGCAGTGTGTTT-3'</td>
</tr>
<tr>
<td>Ppard</td>
<td>5'-TACACAGACAGAGCCCACTC-3'</td>
<td>5'-TACACAGACAGAGCCCACTC-3'</td>
</tr>
<tr>
<td>Rcan1.4</td>
<td>5'-GACAGCCTCTGCTGACATG-3'</td>
<td>5'-GACAGCCTCTGCTGACATG-3'</td>
</tr>
<tr>
<td>Sirt5</td>
<td>5'-CCACGGACACCTATTAAACTCG-3'</td>
<td>5'-GATTCTGGGCTCTGCTACT-3'</td>
</tr>
</tbody>
</table>
2.4 Generation of pressure overload and serial echocardiography

Pressure overload in mice was performed by Lan Mao of the Duke Cardiovascular Physiology Core and was induced using methods previously described (Rockman et al., 1991), except that the suture was placed between the left carotid and the left axillary arteries. Serial echocardiography was performed on conscious mice from all groups with a Vevo 2100 high-resolution imaging system (VisualSonics) as previously described. Mice underwent either a sham or pressure overload procedure in a non-randomized fashion.

2.5 Pressure Volume Loop Analysis

In vivo pressure-volume (P-V) analysis was performed by Lan Mao of the Duke Cardiovascular Physiology Core as previously described (Yoo et al., 2009). Briefly, after bilateral vagotomy, the chest was opened and the pericardium was dissected to expose the heart. A 7-0-suture ligature was placed around the transverse aorta to manipulate loading conditions. A 1.4-Fr pressure-conductance catheter (Millar Instruments, Houston, TX) was inserted retroaortically into the LV to record hemodynamics. Baseline hemodynamic parameters were obtained once the catheter recordings had achieved steady state, usually 3-5 minutes following conductance catheter placement. Load independent parameters were established by generating a series of PV loops with decreasing preload through transient constriction of the inferior vena cava.
Subsequently, parallel conductance (Vp) was determined by 10 µl injection of 15% saline into the right jugular vein to establish the parallel conductance of the blood pool. The derived Vp was used to correct the PV loop data. Data were recorded digitally at 1,000 Hz and analyzed with pressure volume analysis software (PVAN data analysis software version 3.3; Millar Instruments) as previously described (Yoo et al., 2009). Mice that died after receiving anesthesia or became hypotensive during the protocol, suggesting a surgical complication were excluded. Pressure overload mice with a pressure gradient of < 20 mmHg were excluded from the study.

2.6 Metabolomics

2.6.1 Metabolite Extraction

Animals were euthanized by exposure to CO₂ for 5 minutes and immediate excision of the whole heart. Heart was washed in PBS and the left ventricle was dissected and immediately flash frozen. Left ventricle tissue was pulverized using a Bessman Tissue Pulverizer (Spectrum Labs) in liquid nitrogen. 10 to 20 mg of frozen pulverized heart tissue was weighed in an Eppendorf tube and 200 µl of ice cold 80% methanol was added. A glass bead was added and was homogenized using the TissueLyzer (Qiagen) for 2 minutes at 30 Hz. 300 µl ice cold 80% methanol was added, vortexed, and incubated on ice for 10 minutes. Tissue extract was centrifuged at 20,000 x g at 4 °C for 10 minutes. Supernatant containing 2 mg tissue was transferred to a new
Eppendorf tube and dried in vacuum concentrator at room temperature. The dry pellets were reconstituted into 30 µl (per 2 mg tissue) sample solvent (water:methanol:acetonitrile, 2:1:1, v/v) and 3 µl was further analyzed by liquid chromatography-mass spectrometry (LC-MS) by the Juan Liu in Jason Locasale’s lab at Duke University.

2.6.2 LC-MS Method

Ultimate 3000 UHPLC (Dionex) is coupled to Q Exactive Plus-Mass spectrometer (QE-MS, Thermo Scientific) for metabolite profiling. A hydrophilic interaction chromatography method (HILIC) employing an Xbridge amide column (100 x 2.1 mm i.d., 3.5 µm; Waters) is used for polar metabolite separation. Detailed LC method was described previously (Liu et al., 2014) except that mobile phase A was replaced with water containing 5 mM ammonium acetate (pH 6.8). The QE-MS is equipped with a HESI probe with related parameters set as below: heater temperature, 120 °C; sheath gas, 30; auxiliary gas, 10; sweep gas, 3; spray voltage, 3.0 kV for the positive mode and 2.5 kV for the negative mode; capillary temperature, 320 °C; S-lens, 55; scan range (m/z): 70 to 900 for pos mode (1.31 to 12.5 min) and neg mode (1.31 to 6.6 min) and 100 to 1000 for neg mode (6.61 to 12.5 min); resolution: 70000; automated gain control (AGC), 3 × 10⁶ ions. Customized mass calibration was performed before data acquisition.
2.6.3 Metabolomics Data Analysis

LC-MS peak extraction and integration were performed using commercial available software Sieve 2.2 (Thermo Scientific) by Juan Liu in Jason Locasale’s lab at Duke University. The peak area was used to represent the relative abundance of each metabolite in different samples. The missing values were handled as described in previous study (Liu et al., 2014). Data was uploaded to MetaboAnalyst 3.0 (Xia Lab, McGill University, Quebec, Canada) for further downstream analysis by myself (Xia and Wishart, 2016; Xia et al., 2015).

2.7 CST Succinyl-Proteomics

2.7.1 Sample Preparation

Animals were euthanized by exposure to CO₂ for 5 minutes and immediate excision of the whole heart. Heart was washed in PBS and the left ventricle was dissected and immediately flash frozen. Samples were sent on dry ice to Cell Signaling Technologies for further preparation and analysis. Sample preparation, LC-MS/MS, and data analysis were performed by Hongbo Gu (Cell Signaling Technology, Boston, MA). Samples were analyzed using the PTMScan method as previously described (Rush et al., 2005; Stokes et al., 2015). Cellular extracts were prepared in urea lysis buffer, sonicated, centrifuged, reduced with DTT, and alkylated with iodoacetamide. 15 mg total protein for each sample was digested with trypsin and purified over C18 columns for
enrichment with the Succinyl-Lysine Motif Antibody (#13764). Enriched peptides were purified over C18 STAGE tips (Rappsilber et al., 2003). Enriched peptides were subjected to secondary digest with trypsin and second STAGE tip prior to LC-MS/MS analysis.

2.7.2 LC-MS/MS Method

Replicate injections of each sample were run non-sequentially for each enrichment. Peptides were eluted using a 120-minute linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min. Tandem mass spectra were collected in a data-dependent manner with an LTQ Orbitrap VELOS mass spectrometer running XCalibur 2.0.7 SP1 using a top-twenty MS/MS method, a dynamic repeat count of one, and a repeat duration of 30 seconds. Real time recalibration of mass error was performed using lock mass (Olsen et al., 2005) with a singly charged polysiloxane ion m/z = 371.101237.

2.7.3 Data Analysis

MS/MS spectra were evaluated using SEQUEST and the Core platform from Harvard University (Eng et al., 1994; Huttlin et al., 2010; Villén et al., 2007). Files were searched against the NCBI *mus musculus* FASTA database updated on April 29, 2015. A mass accuracy of +/-5 ppm was used for precursor ions and 1.0 Da for product ions. Enzyme specificity was limited to trypsin, with at least one LysC or tryptic (K- or R-containing) terminus required per peptide and up to four mis-cleavages allowed. Cysteine carboxamidomethylation was specified as a static modification, oxidation of
methionine and succinylation on lysine residues were allowed as variable modifications. Reverse decoy databases were included for all searches to estimate false discovery rates, and filtered using a 5% FDR in the Linear Discriminant module of Core. Peptides were also manually filtered using a +/- 5 ppm mass error range and reagent-specific criteria. Results were filtered to include only peptides containing at least one Succinyl-lysine residue. All quantitative results were generated using Progenesis V4.1 (Waters Corporation) to extract the integrated peak area of the corresponding peptide assignments. Accuracy of quantitative data was ensured by manual review in Progenesis or in the ion chromatogram files. I received the searched data from CST and further analyzed the data for inclusion in this dissertation. Ingenuity Pathway Analysis software (Qiagen) was used to determine pathways enriched with succinylated proteins.

2.8 TMT-Labeled Succiny1-Proteomics

I performed the following under the guidance of Paul Grimsrud of the Proteomics Laboratory at the Duke Molecular Physiology Institute: sample preparation and lysis, TMT labeling, succinyl-lysine IP, and input fractionation and quantification. Paul Grimsrud performed the mass spec and searched the data. I analyzed the searched data and generated figures for inclusion in this dissertation.
2.8.1 Sample Preparation and Lysis

Animals were euthanized by exposure to CO$_2$ for 5 minutes and immediate excision of the whole heart. Heart was washed in PBS and the left ventricle was dissected and immediately flash frozen. Left ventricle tissue was pulverized using a Bessman Tissue Pulverizer (Spectrum Labs) in liquid nitrogen and approximately 20 mg of tissue was weighed out for further sample preparation. 300 µl of urea lysis buffer (8 M urea, 50 mM pH 8.0 Tris, 40 mM NaCl, 2 mM MgCl$_2$, 10 mM nicotinamide, 10 µM TSA, protease inhibitors (Roche cOmplete Ultra)) was added to each sample and disrupted with the TissueLyzer (Qiagen) for one minute at 30 Hz. Samples were frozen on dry ice and thawed at 32 °C for three freeze-thaw cycles. Samples were sonicated with a pencil-tip probe sonicator at power level 3, 3 bursts for 5 seconds each. Samples were centrifuged at 10,000 x g for 10 minutes at 4 °C. Supernatant was placed in clean tube and BCA assay (Sigma) was performed to determine protein concentration. 500 µg of each sample were diluted in 200 µl urea lysis buffer.

Samples were reduced by adding 5 mM dithiothreitol (DTT) and incubating at 32 °C for 30 minutes. Samples were alkylated by adding 15 mM iodoacetamide (IAM) and incubating for 30 minutes at room temperature in the dark. This reaction was quenched by adding DTT to a final concentration of 15mM. 500µg of LysC (Wako) was added to each sample and digested for four hours at 32 °C. Samples were diluted to 1.5 M urea and 10 µg sequence grade trypsin (Promga) were added. Samples were digested
overnight at 32 °C. Samples were acidified to 0.5% v/v trifluoroacetic acid (TFA) and spun down at room temperature. Samples were cleaned by loading and eluting from a 50 mg tC18 Sep-Pak SPE column (Waters) Samples were dried on a Speed Vac overnight on the no-heat setting.

2.8.2 TMT Labeling

Samples were resuspended in 100 µl of 200 mM triethylammonium bicarbonate (TEAB). Labeling reagents (Thermo TMT10plex Kit, 90110) were resuspended in 50µl acetonitrile (ACN). Labeling reagents were added to the sample tubes and shook for four hours at room temperature. Reactions were quenched by adding 0.8 µl of hydroxylamine and shaking for 15 minutes at room temperature. All samples were combined into a single tube. 5% of the sample was removed for input quantification. Sample and input were dried on a Speed Vac overnight on the no-heating setting. Combined sample was desalted on a 100 mg tC18 Sep-Pak SPE column (Waters), eluted, and dried overnight on a Speed Vac on the no-heat setting.

2.8.3 Succinyl-Lysine IP

Beads conjugated with succinyl-lysine antibody (CST, PTMscan Succinyl-Lysine Motif, 13764) were washed two times in ice cold PBS. All wash steps include spinning the bead slurry down at 2,000 x g at 4 °C for 30 seconds and carefully removing the supernatant. Beads were transferred to a new tube and washed two more times with ice cold PBS. TMT-labeled sample was resuspended in 1.4 mL 1X IAP buffer (CST,
PTMscan Succinyl-Lysine Motif, 13764) and centrifuged at 10,000 x g at 4 °C for 5 minutes. The supernatant was added to the washed succinyl-lysine antibody beads and incubated on a rotator overnight at 4 °C.

Sample was centrifuged at 2,000 x g at 4 °C for 30 seconds and flow through was saved. Beads were washed two times with 1X IAP with gentle mixing by inversion in between washes. Beads were washed three times with milli-Q H₂O and transferred to a new tube. Succinyl peptides were eluted from the beads by adding 100 µl of 0.15% TFA and letting sit at room temperature for 10 minutes with gentle mixing every 2-3 minutes. Sample was centrifuged at 2,000 x g for 30 seconds and supernatant transferred to new tube. This elution step was repeated once and sample was brought to a final concentration of 0.5% TFA. Sample was cleaned by loading and eluting from a 50 mg tC18 Sep-Pak SPE column (Waters). Sample was dried on a Speed Vac overnight on the no-heat setting. Sample was resuspended in 22.5 µl 0.1% formic acid (FA), loaded into an auto-sampler tube, and stored at -80°C until ready for mass spec analysis.

2.8.4 Input fractionation and quantification

Input sample was resuspended in 0.5 mL of 0.1% TFA and diluted to 0.33 mg/mL in 0.1% TFA. Fractionation columns (Pierce, High pH Reversed-Phase Peptide Fractionation Kit, 84868) were conditioned according to the manufacturer’s instructions. 300 µl of sample was loaded onto a fractionation column and centrifuged at 3,000 x g for 2 minutes at room temperature. Sample was washed with 300 µl water followed by 300
µl of 5% ACN, 0.1% TFA. Sample was eluted in 8 fractions containing increasing amount of ACN. Fractions were dried in Speed Vac overnight on no heat setting.

Sample fractions were resuspended in 10 µl of 0.1% FA. Peptide fractions were quantified using Pierce Quantitative Colorimetric Peptide Assay (ThermoScientific, 23275) and sample fractions were brought to a final concentration of 0.15 mg/mL in 0.1% FA. Fractionation samples were loaded into an auto-sampler tube, and stored at -80°C until ready for mass spec analysis.

2.8.5 LC-MS/MS Method

All samples were subjected to nanoLC-MS/MS analysis using a nano-Acquity UPLC system (Waters) coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ionization source. Succinyl-peptides were run in technical duplicates. For each injection, the samples was first trapped in a Symmetry C16 20mm x 180mm trapping column (5uL/min at 99.89/0.1 v/v water/acetonitrile, after which the analytical separation was performed over a 90 minute gradient (flow rate of 400 nanoliters/minute) of 3 to 30% acetonitrile using a 1.7um Acquity BEH130 C18 75um x 250mm column (Waters Corp.), with a column temperature of 55°C. MS\(^1\) (precursor ions) was performed at 70,000 resolution, with an AGC target of 1x10\(^6\) ions and a maximum injection time of 60ms. MS\(^2\) spectra (product ions) were collected by data-dependent acquisition (DDA) of the top 20 most abundant precursor ions with a charge greater than 1 and less than 8 per MS\(^1\) scan, with dynamic
exclusion enabled for a window of 30 seconds. Precursor ions were filtered with a 1.2m/\textit{z} isolation window and fragmented with a normalized collision energy of 30. MS$^2$ scans were performed at 17,500 resolution, with an AGC target of $1 \times 10^5$ ions and a maximum injection time of 60ms. The input material fractions were analyzed in singlicate, with 2uL injections, run as above but with the gradient lengthened to 180 minutes.

2.8.6 Data Analysis

Raw LC-MS/MS data were processed in Proteome discoverer v2.1 with service pack 1 (PD2.1, SP1, Thermo Fisher Scientific), using both the Sequest HT and MS Amanda search engines. Data were searched against the UniProt mouse complete proteome database of reviewed (Swiss-Prot) and unreviewed (TrEMBL) proteins. Considering each data type (succinyl and input) separately, peptide spectral matches (PSMs) from each search algorithm were filtered to a 1% false discovery rate (FDR) using the percolator node of PD2.1. For succinyl data, site localization probabilities were determined for all modifications using the ptmRS algorithm. PSMs were grouped to unique peptides while maintaining a 1% FDR at the peptide level and using a 95% site localization threshold for succinyl modifications. Peptides from all samples (succinyl and input) were grouped to proteins together using the rules of strict parsimony and proteins were filtered to 1% FDR using the Protein FDR Validator node of PD2.1. Reporter ion intensities for all PSMs having co-isolation interference below 0.5 (50% of the ion current in the isolation window) and average reporter S/N>1 for all reporter ions
were summed together at the peptide group and protein level, but keeping quantification for each data type (succinyl, input) and each experimental group separate. Peptides shared between protein groups were excluded from protein quantitation calculations.

Protein and peptide groups tabs in the PD2.1 results were exported as tab delimited .txt. files, opened in Microsoft EXCEL, and analyzed as described previously (McDonnell et al., 2016). First, peptide group reporter intensities for each peptide group in the input material were summed together for each TMT channel. Each channel’s sum was divided by the average of all channels’ sums, resulting in channel-specific loading control normalization factors to correct for any deviation from equal protein/peptide input into the nine-sample comparison. Reporter intensities for peptide groups from both the succinyl fractions and for proteins from the input fraction were divided by the loading control normalization factors for each respective TMT channel. Analyzing the succinyl-peptide and protein datasets separately, all loading control-normalized TMT reporter intensities were converted to log₂ space, and the average value from the nine samples was subtracted from each sample-specific measurement to normalize the relative measurements to the mean. For protein-level quantification, only Master Proteins—or the most statistically significant protein representing a group of parsimonious proteins containing common peptides identified at 1% FDR were used for quantitative comparison. PTM level measurements (succinyl) were calculated both alone
(referred to as *abundance*) and with normalization to any change in the corresponding Master Protein (referred to as *relative occupancy*), calculated by subtracting Log, Master Protein values from PTM-containing peptide quantitation values on a sample-specific basis. Adjusted p values were calculated using Benjamini-Hochberg correction and a 10% FDR was used to determine statistical significance.

### 2.9 Statistical Analysis

Statistical analysis for all studies was performed using Prism6. A two-sided Grubb’s test was used to determine outliers on data sets with n>6. Outliers were removed from data; no more than one outlier per group was removed. Outliers were only found in the pressure volume loop with inferior vena cava occlusion hemodynamic data. For studies with 4 conditions (WT sham, WT TAC, SIRT5KO sham, and SIRT5KO TAC), a two-way ANOVA with multiple comparisons was performed. Bonferroni correction was used to correct for multiple comparisons. A corrected p-value of ≤ 0.05 was considered significant. For studies with only WT and SIRT5KO comparisons, a student’s t-test was performed and a p-value of ≤ 0.05 was considered significant. Strong trends (p-value ≤ 0.1 are noted throughout the dissertation). Please refer to sections 2.7.3 and 2.8.6 for data searching and analysis methods (including FDR values) of large proteomic data sets.
3. Whole-Body SIRT5KO Mouse Studies

3.1 Introduction

Sirtuin 5 (SIRT5) is one of three mitochondrial proteins that belongs to the sirtuin family of NAD+-dependent deacylases. Mitochondrial sirtuins (SIRT3-5) control metabolism in physiological and pathophysiological conditions by their deacylation activity (Anderson et al., 2014; Hirschey and Zhao, 2015). SIRT5 possesses demalonylase, desuccinylase (Du et al., 2011; Peng et al., 2011), and deglutarylase activity (Tan et al., 2014), while SIRT3 is primarily a lysine deacetylase (Anderson et al., 2014) and SIRT4 is a potent lysine deglutarylase, demethylglutarylase and dehydroxymethylglutarylase (Anderson et al., 2017). The protein acylation profile in SIRT5KO mice has been characterized by several proteomic studies with the goal of identifying SIRT5 targets (proteins with increased sites of lysine acylation in the SIRT5KO mouse compared to wild-type controls) and the role of SIRT5 in regulating metabolism. For example, analyses of SIRT5KO mouse liver succinyl-proteomic datasets identified many proteins in metabolic pathways enriched with protein lysine succinylation including enzymes involved in fatty acid oxidation, branched chain amino acid (BCAA) degradation, and the TCA cycle (Park et al., 2013; Rardin et al., 2013a). Within these pathways, specific targets of SIRT5 include pyruvate dehydrogenase (PDH), succinate dehydrogenase (SDH) (Park et al., 2013), and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) (Rardin et al., 2013a), to name a few. SIRT5-mediated desuccinylation has been reported
to repress activity of PDH (Park et al., 2013) and enhance activity of HMGCS2 (Rardin et al., 2013a). SDH activity has been reported to be both repressed by desuccinylation (Boylston et al., 2015; Rardin et al., 2013a), as well as enhanced (Zhang et al., 2017). Aside from these targets, hundreds of sites of lysine succinylation (Rardin et al., 2013a), malonylation (Nishida et al., 2015) and glutarylation (Tan et al., 2014) lack functional characterization. Thus, further work needs to be done to fully understand how sirtuins regulate their cellular targets. Studies investigating the precise mechanisms by which SIRT5 regulates metabolism and function are mainly focused on analyzing the consequence of protein acylation and deacylation on identified SIRT5 targets (Nakamura et al., 2012; Polletta et al., 2015). However, despite the discovery of several SIRT5 substrates in mouse liver tissue, no strong, liver-specific phenotypes in SIRT5KO mice have been found. Indeed, overall phenotypes in mice lacking SIRT5 are generally subtle.

SIRT5 protein and mRNA is highly expressed in mouse and human heart compared to other tissues (Nishida et al., 2015). The heart is a highly metabolic tissue and relies predominately on oxidative metabolism to produce sufficient ATP for cardiac muscle contraction. Previous studies demonstrated that cardiac hypersuccinylation occurs in the absence of SIRT5 and that enzymes in several metabolic pathways are primarily enriched with succinylation (Boylston et al., 2015; Sadhukhan et al., 2016). Despite strong hyperacylation signals, characterization of the SIRT5KO mouse reveals no substantial metabolic phenotypes (Yu et al., 2013) and only a mild decrement in
cardiac function that is apparent in early adulthood (Sadhukhan et al., 2016). Based on current knowledge, many metabolic enzymes are hyperacylated in the absence of SIRT5, but few physiological consequences of SIRT5 ablation under normal conditions have been found.

Consistent with the notion that sirtuins are stress response proteins, studies to elucidate the role of SIRT5 in cardiac physiology have revealed that physiological stress is necessary to reveal a phenotype in SIRT5KO mice. For example, the acute stress of cardiac ischemia-reperfusion results in an increased infarct area in mice lacking SIRT5 (Boylston et al., 2015). Additionally, aging SIRT5KO mice to 39 weeks results in increased cardiac hypertrophy and reduced cardiac function (Sadhukhan et al., 2016). Increased activity of SDH and decreased activity of hydroxyacyl-CoA dehydrogenase alpha subunit (HADHA) via increased hypersuccinylation in the SIRT5KO mouse heart were identified as mechanisms contributing to these cardiac phenotypes. Together, these studies demonstrate that SIRT5 regulates cardiac metabolism via its desuccinylase activity. Despite this progress, the overall role of SIRT5 on cardiac metabolism and function requires further study. Thus, we considered interrogating the role of SIRT5 in the heart by using a model of chronic cardiac stress, namely a pressure-overload-induced hypertrophy model.

Pressure overload causes an increase in ventricular wall stress and cardiomyocyte growth (Abel and Doenst, 2011; Dorn, 2007) that results in maladaptive
cardiac hypertrophy (Frey and Olson, 2003). This form of remodeling precedes the development of cardiac dysfunction, which is closely associated with shifts in cardiac metabolism. Classically, these metabolic shifts include both a decrease in the contribution of fatty acid oxidation to ATP production, and an increase in the contribution of glycolysis to ATP production. Ultimately, oxidative metabolism decreases and ATP production is not sufficient to meet the energetic demands of the heart and failure occurs. Given the decline in oxidative metabolism in cardiac hypertrophy, and that identified targets of SIRT5 are enzymes in oxidative metabolic pathways, we predicted that SIRT5 might play a role in controlling metabolism during the transition to cardiac dysfunction. Thus, we set out to characterize cardiac metabolism and physiology in whole body SIRT5KO mice.

3.2 Results

3.2.1 Chronic TAC results in significantly increased mortality in SIRT5KO mice

Transverse aortic constriction (TAC) is a well-characterized animal model of pressure-overload-induced hypertrophy (Rockman et al., 1991), wherein metabolic shifts away from oxidation occur that are associated with the onset of metabolic dysfunction. SIRT5KO mice and wild-type (WT) controls (10-12 weeks of age) underwent TAC and were monitored for 16 weeks. In response to TAC, median survival was starkly decreased in SIRT5KO mice compared to WT controls (2.4 versus 10.9 weeks post-TAC;
Figure 6 A). No death was observed in the sham control groups for either genotype (data not shown). These findings suggest that SIRT5KO mice progress to cardiac dysfunction at an accelerated rate upon TAC-induced cardiac stress compared to controls.

Serial echocardiography was used to measure wall thickness (WTh) (interventricular septum wall thickness + posterior wall thickness) and fractional shortening (FS) [(EDD-ESD)/EDD*100)] before TAC surgery and 2-, 4-, 8-, 12-, and 16-weeks post-TAC surgery. Due to the significant, progressive mortality in the SIRT5KO group, it was not possible to perform appropriate statistical analyses of this experiment. However, a trend appeared for greater wall thickness in the SIRT5KO mice at 2- and 4-weeks post-TAC (Figure 6 B). No differences were measured in cardiac fractional shortening between the WT and SIRT5KO mice at any time point (Figure 6 C). Based on these findings, and to overcome the progressive mortality, further examination of cardiac physiology was restricted to the 4-week post-TAC time point in WT and surviving SIRT5KO mice.
WT and SIRT5KO mice were exposed to pressure overload for 16 weeks via TAC surgery. (A) Survival of the SIRT5KO mice was significantly reduced. Mantel-Cox test. (B) Percent change wall thickness appeared to be increased in the SIRT5KO in the early stages of a 16-week chronic TAC study. (C) Percent change in fractional shortening decreased in both wild-type and SIRT5KO animals over the course of the study with no apparent genotypic differences. n=10 WT TAC, and n=9 SIRT5KO TAC at start of study. Males, 10-12 weeks of age at start of study.

Wall thickness and fractional shortening were measured by echocardiogram pre-TAC and 4-weeks post-TAC, and these data were combined with data at the same time.
points from the initial 16-week study to assess cardiac morphology and function. Concentric hypertrophy was increased in SIRT5KO TAC mice compared to WT TAC mice after 4 weeks of TAC (Figure 7 A and B). As expected, wall thickness (including both measurements of interventricular septum wall (IVSW) and posterior wall (PW) thickness) increased with TAC in both genotypes compared to their respective sham controls (Figure 7 A). No significant differences in fractional shortening were seen between WT and SIRT5KO mice 4 weeks post-TAC (Figure 7 C and D). Further, we found no differences in wall thickness or fractional shortening between the WT and SIRT5KO sham conditions (Figure 7 A-D). Consistent with echocardiographic measurements of concentric hypertrophy, we observed an increase in left ventricle (LV) weight/body weight with TAC in both genotypes compared to their sham controls, indicative of the development of left ventricle cardiac hypertrophy (Figure 7 E).

Although we observed a significant increase in wall thickness between the WT TAC and SIRT5KO TAC group, there was no difference in the left ventricle to body weight ratio between these two groups. This suggests that WT and SIRT5KO mice had a similar hypertrophic response to pressure overload. There are a number of factors that could explain the increased left ventricle wall thickness we observed between WT TAC and SIRT5KO TAC animals using echocardiography.

A variety of technical and physiological factors influence the accuracy of echocardiogram readings. Technically, the position of the ultrasound transducer may
overestimate left ventricle internal dimensions and wall thickness if the beam is not parallel to the LV minor axis, but angled obliquely from it (Popp et al., 1975). This factor may be further compounded when serial echocardiograms are made on the same animal, as the position of the ultrasound transducer should be identical to previous readings (Wallerson and Devereux, 1987). In our study, serial echocardiograms were taken pre-TAC and 4-weeks post-TAC. Inaccurate position of the ultrasound could explain some of the artificially increased wall thickness observed in our echocardiogram readings. Additionally, the reproducibility of echocardiogram readings is greatest when read by 2 readers on 2 separate occasions each, or 3 readers reading each echocardiogram once (Clark et al., 1980). In this study, echocardiogram readings were only performed by one reader, which could decrease accuracy of the readings. However, the reader was blinded to the genotypes of the animals, so it is unlikely that this factor accounts for the enhanced wall thickness observed in the SIRT5KO TAC group.

Physiological factors including heart rate, preload, and afterload respond to environmental factors such as stress and can influence echocardiogram readings. In terms of wall thickness, an increase in heart rate can lead to an overestimation of left ventricle wall thickness in echocardiogram readings, including both left ventricle posterior wall thickness and septum wall thickness (DeMaria et al., 1979). However, when we compare heart rate at the time of echocardiogram during the pre-TAC reading and 4-week post-TAC reading, we observe no differences in heart rate between groups.
(data not shown), suggesting that this may not be a main reason for increased wall thickness in the SIRT5KO TAC compared to WT TAC group. Preload can be changed by dehydration (resulting in a lower blood volume) and could also cause the impression of hypertrophy (Di Segni et al., 1997). We are not able to experimentally determine hydration status in these animals post-hoc. Interestingly, the physiological effects that could lead to an apparent increase in wall thickness seems to be an effect of SIRT5.

Given these caveats that can influence accuracy of echocardiogram readings, it is necessary to confirm our observations of increased wall thickness in SIRT5KO TAC mice compared to WT TAC mice with other measurements of cardiac hypertrophy. The ratio of left ventricle weight to body weight demonstrated that there was no difference between WT TAC and SIRT5KO TAC groups. Body weight can decrease with TAC, and thus a more accurate measurement of this could be left ventricle weight to tibia length. Since we observe no differences in body weight, this suggests that the ratio of left ventricle weight to body weight is an accurate representation of left ventricle hypertrophy. Finally, hypertrophy is a result of an increase in cardiomyocyte size. Measuring cardiomyocyte cell size by histology and microscopy in these groups of animals would provide further evidence for any differences in hypertrophy between WT TAC and SIRT5KO TAC hearts. Based on the incongruent echocardiogram data and left ventricle weight data, we cannot conclude that there is exaggerated hypertrophy in the SIRT5KO TAC compared to WT TAC animals 4-weeks post-TAC. Given the variability
associated with echocardiography in terms of technical and physiologic factors, it is possible that the echocardiogram data does not accurately represent cardiac morphology and hypertrophy will need to be evaluated using other techniques in order to draw definite conclusions.

Consistent with these morphological changes, two molecular markers of hypertrophy, namely Anf and Bnp, trended towards increased in both WT and SIRT5KO animals after 4 weeks of TAC (Figure 7 F). Additionally, we found markers of cardiac fibrosis (Col3a1 and Col1a1) to be increased in the SIRT5KO TAC compared to SIRT5KO sham mice (Figure 7 F). Col1a1 was also higher in the SIRT5KO TAC compared to WT TAC animals. The mechanical stress of TAC activates several primary pathways including the mitogen-activated protein kinase (MAPK) pathway and activation of calcineurin (Ruwhof and van der Laarse, 2000). A main effect of hypertrophic signaling is increased phosphorylation of NFAT that leads to its translocation to the nucleus and transcription of hypertrophic genes. We interrogated the expression of Rcan1.4, a target gene of NFAT signaling (van Oort et al., 2010) and found that expression of this gene trended towards increased in both TAC conditions, with a significant increase in the SIRT5KO TAC compared to sham condition (Figure 7 G). This data is consistent our other findings of exacerbated hypertrophy in SIRT5KO mice with TAC. Together, these observations suggest that WT and SIRT5KO mice develop hypertrophy 4-weeks post-TAC, with no difference in genotype. Therefore, it does not appear that an exaggerated

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response to TAC is responsible for decreased survival observed in the SIRT5KO TAC compared to WT TAC group.

Figure 7: SIRT5KO and WT mice develop hypertrophy 4-weeks post-TAC

Measurements of cardiac morphology at 4-weeks post-TAC: (A) Wall thickness (WTh), interventricular septum wall (IVSW) thickness, and posterior wall (PW) thickness. (B) Percent change of values in panel A. (C) Fractional shortening (FS), end diastolic diameter (EDD), and end systolic diameter (ESD). (D) Percent change of values in C. n=16 WT Sham, n=13 WT TAC, n=13 SIRT5KO sham, n=16 SIRT5KO TAC. Combined results from 6 independent experiments including 4-week time point from figure 1. Males, 12-21 weeks of age at 4 weeks TAC, A-D. Boxplots depict the interquartile range with whiskers plotted to the minimum and maximum values. The horizontal line within the box is the median value and the “+” is the mean value. (E) Left ventricle weight normalized to body weight at 4-weeks post-TAC. n=12 WT Sham, n=14 WT
TAC, n=10 SIRT5KO sham, n=18 SIRT5KO TAC. Combined results from 5 independent experiments. Males, 12-21 weeks of age at 4 weeks TAC. (f) Cardiac mRNA expression of Anf, Bnp, Col3a1 and Col1a1 relative to 36B4. Values are mean ± SE. (G) Rcan1.4 relative to 36B4. Values are mean ± SE. n=3 WT Sham, n=5 SIRT5KO Sham, n=4 WT TAC, n=7 SIRT5KO TAC. Males, 18-19 weeks of age at 4-weeks post-TAC. Two-way ANOVA with multiple comparisons, Bonferroni correction. * p ≤ 0.05; Effect of TAC if no significance in multiple comparisons tests. + p ≤ 0.05

### 3.2.2 SIRT5KO and WT mice have impaired systolic function 4-weeks post-TAC

To further investigate the functional impact of SIRT5 ablation on the heart, we performed pressure-volume loop analyses after 4 weeks of TAC (Figure 9 A-D). Systolic function worsened in both TAC conditions as indicated by a decrease in cardiac output, and trends of decreased stroke volume and dP/dt\text{max} (Table 2). With development of cardiac hypertrophy, ejection fraction was maintained in WT TAC mice, and trended towards decreased in SIRT5KO TAC compared to SIRT5KO sham mice (p=0.07) (Fig. 3E). Load-independent measures of contractility, specifically end-systolic pressure volume relationship (ESPVR) linear slope and maximum elastance (Emax), showed increases in the SIRT5KO TAC animal compared to their sham controls (Table 3). Measures of active relaxation, such as tau and dP/dt\text{min} (Table 2), and passive relaxation, such as the end diastolic pressure volume relationship (EDPVR) (Table 3), suggests both prolonged active relaxation and elevated ventricular stiffness in both WT and SIRT5KO mice. Overall, WT and SIRT5KO mice demonstrate pathologic hypertrophy in response to pressure overload, with minor abnormalities in both cardiac
relaxation and performance. While there appear to be mild decrements in cardiac function in both WT and SIRT5KO mice with TAC, there are no differences in cardiac function when comparing WT TAC and SIRT5KO TAC animals. These data suggest that cardiac dysfunction may not be the cause of increased mortality in the SIRT5KO TAC group.

Table 2: Baseline Hemodynamic Parameters (WT and SIRT5KO sham and TAC).

Statistical comparisons were made using a Two-Way ANOVA with Bonferroni correction for multiple comparisons. Data are presented as mean ± SE. *p≤0.1 vs. sham of same genotype. **p≤0.05 vs. sham of same genotype.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type Sham (n=6)</th>
<th>SIRT5KO Sham (n=9)</th>
<th>Wild Type TAC (n=7)</th>
<th>SIRT5KO TAC (n=11)</th>
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</thead>
<tbody>
<tr>
<td>Heart Rate (BPM)</td>
<td>460.0 ± 5.9</td>
<td>451.6 ± 18.1</td>
<td>378.6 ± 22.6**</td>
<td>392.6 ± 41.3**</td>
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<tr>
<td>LVESP (mm Hg)</td>
<td>118.2 ± 16.1</td>
<td>120.4 ± 8.6</td>
<td>166.4 ± 9.5**</td>
<td>197.0 ± 7.3**</td>
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<tr>
<td>LVEDP (mm Hg)</td>
<td>8.78 ± 2.02</td>
<td>6.60 ± 1.55</td>
<td>11.57 ± 1.16</td>
<td>12.28 ± 2.43</td>
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<tr>
<td>Stroke Volume (µl)</td>
<td>27.72 ± 1.74</td>
<td>21.35 ± 2.78</td>
<td>19.29 ± 2.46</td>
<td>15.25 ± 1.74</td>
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<tr>
<td>Ejection Fraction (%)</td>
<td>62.12 ± 5.75</td>
<td>56.97 ± 6.47</td>
<td>48.97 ± 9.29</td>
<td>37.16 ± 3.53*</td>
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<tr>
<td>Cardiac Output (µl/min)</td>
<td>12747 ± 813.0</td>
<td>9828 ± 1532.0</td>
<td>7451 ± 1190**</td>
<td>6036 ± 748.4*</td>
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<tr>
<td>Stroke Work (mmHg*µl)</td>
<td>2522 ± 203.4</td>
<td>2085 ± 287.3</td>
<td>2527 ± 396.6</td>
<td>2045 ± 301.8</td>
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<tr>
<td>dP/dt_max (mm Hg/s²)</td>
<td>10259 ± 1099</td>
<td>10377 ± 812.1</td>
<td>8152 ± 830.0</td>
<td>8476 ± 745.5</td>
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Systolic Function Parameters

<table>
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<th>Wild Type Sham (n=6)</th>
<th>SIRT5KO Sham (n=9)</th>
<th>Wild Type TAC (n=7)</th>
<th>SIRT5KO TAC (n=11)</th>
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<tr>
<td>dP/dt_min (mm Hg/s)</td>
<td>-8502 ± 841.6</td>
<td>-8520 ± 486.8</td>
<td>-7865 ± 694.7</td>
<td>-8845 ± 831.34</td>
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<tr>
<td>Tau (ms) (Weiss)</td>
<td>7.88 ± 0.38</td>
<td>7.44 ± 0.38</td>
<td>9.41 ± 0.76</td>
<td>9.47 ± 1.05</td>
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<tr>
<td>Tau (ms) (Glantz)</td>
<td>12.77 ± 1.22</td>
<td>11.81 ± 0.96</td>
<td>14.29 ± 0.95</td>
<td>14.31 ± 1.32</td>
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Diastolic Function Parameters
Table 3: Load Independent Hemodynamic Measures (WT and SIRT5KO sham and TAC).

Parameters of LV compliance (linear and quadratic derived EDPVR) and LV contractility (linear and quadratic derived ESPVR, PRSW, dP/dtmax vs EDV and Emax); Data represented as mean ± SE; Statistical comparisons made with a Two-Way ANOVA with Bonferroni correction for multiple comparisons. *p≤0.1 vs. sham of same genotype; **p≤0.05 vs. sham of same genotype; EDPVR=End Diastolic Pressure Volume Relationship; ESPVR=End Systolic Pressure Volume Relationship; a= coefficient of curvilinearity; Vo=Volume intercept; E’max= maximum slope of quadratic ESPVR; PRSW= Preload Recruitable Stroke Work; Emax=maximal elastance.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type Sham (n=6)</th>
<th>SIRT5KO Sham (n=7)</th>
<th>Wild Type TAC (n=9)</th>
<th>SIRT5KO TAC (N=11)</th>
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<tr>
<td><strong>Compliance Parameters</strong></td>
<td></td>
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<tr>
<td>EDPVR (Linear) Slope</td>
<td>0.19 ± 0.07</td>
<td>0.27 ± 0.06</td>
<td>0.36 ± 0.06</td>
<td>0.63 ± 0.18</td>
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<tr>
<td>EDPVR (Linear) Intercept</td>
<td>-0.41 ± 0.99</td>
<td>-1.40 ± 1.01</td>
<td>-7.28 ± 5.00</td>
<td>-8.27 ± 6.82</td>
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<td>EDPVR (Quadratic) b coefficient</td>
<td>0.043 ± 0.006</td>
<td>0.071 ± 0.011</td>
<td>0.076 ± 0.012</td>
<td>0.104 ± 0.018</td>
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<tr>
<td>EDPVR (Quadratic) a coefficient</td>
<td>1.606 ± 0.542</td>
<td>0.866 ± 0.111</td>
<td>0.619 ± 0.315</td>
<td>0.482 ± 0.244</td>
</tr>
</tbody>
</table>

| **Contractility Parameters** |                      |                     |                     |                     |
| ESPVR (Linear) Slope | 3.16 ± 0.55          | 5.46 ± 1.11         | 6.93 ± 1.01         | 10.13 ± 1.59**      |
| ESPVR (Linear) Intercept | -19.63 ± 5.77       | -8.54 ± 3.92        | 3.35 ± 7.88**       | 6.65 ± 4.47         |
| ESPVR (Quadratic) a | -0.10 ± 0.06         | -0.23 ± 0.08        | -0.22 ± 0.07        | -0.38 ± 0.15        |
| ESPVR (Quadratic) b | -10.27 ± 4.61        | -2.12 ± 2.95        | 10.96 ± 6.34**      | 11.76 ± 4.23*       |
| ESPVR (Quadratic) E’max | 6.20 ± 1.77          | 9.50 ± 1.70         | 12.51 ± 1.19        | 19.02 ± 3.84*       |
| PRSW Slope | 52.66 ± 4.78         | 61.51 ± 4.81        | 89.52 ± 7.94*       | 89.12 ± 12.95       |
| PRSW Intercept | -4.01 ± 4.72         | 2.52 ± 2.98         | 16.49 ± 6.57*       | 16.36 ± 5.20        |
| dP/dtmax vs EDV slope | 243.91 ± 29.47       | 277.42 ± 50.95      | 161.99 ± 17.53      | 252.92 ± 44.91      |
| dP/dtmax vs EDV intercept | 0.58 ± 4.05          | -2.79 ± 3.64        | 0.57 ± 5.73         | 1.30 ± 5.10         |
| Emax | 4.79 ± 0.76          | 6.50 ± 0.69         | 11.03 ± 1.40        | 15.59 ± 2.49**      |

Next, we analyzed signaling pathways that are activated with cardiac hypertrophy to determine if aberrant signaling may be responsible for increased mortality in the SIRT5KO TAC condition. We analyzed activation of mTOR by probing for phosphorylation of 4EBP1 and found a trend of increased phosphorylation of 4EBP1 in both WT and SIRT5KO TAC condition with no differences in genotype (Figure 8 A
and B). Beta-adrenergic receptor signaling is often a secondary signaling response with pressure overload induced hypertrophy (Ruwhof and van der Laarse, 2000), and is dysfunctional in later stages of heart failure (Lamba and Abraham, 2000). During activation of beta-adrenergic receptor signaling, auto-phosphorylation of CaMKII occurs at Thr286 (Erickson, 2014). We examined phosphorylation of CaMKII and found that there was an increase in phosphorylation of CaMKII in the SIRT5KO TAC compared to sham condition (Figure 8 C and D). While we observed high variability in the WT TAC condition, we found a trend of increased CaMKII phosphorylation. Additionally, we looked at the mRNA expression of beta-adrenergic receptor 1 (Adbr1) and beta-adrenergic receptor 2 (Adbr2) because down-regulation of beta-adrenergic receptors occurs with heart failure (Engelhardt et al., 1996). We found a decrease in Adbr1 in the SIRT5KO TAC compared to sham condition, and a trend of decreased Adbr1 in the WT TAC compared to sham condition (Figure 8 E). These findings are consistent with dysfunctional beta-adrenergic receptor signaling in the WT and SIRT5KO TAC conditions. However, we found no marked differences between the WT and SIRT5KO TAC condition.
Figure 8: Cardiac hypertrophy signaling is similar in WT and SIRT5KO TAC conditions

Left ventricle tissue was isolated and flash frozen after 4 weeks of TAC or sham surgery for further downstream analysis. (A) Western blot of phospho-4EBP1 and total 4EBP1. (B) Quantification of blot, normalized to total protein, boxplots. (C) Western blot of phospho-CaMKIIα and total CaMKIIα. (D) Quantification of blot, normalized to total protein, boxplots. (E) Cardiac mRNA expression of Adbr1 and Adbr2 relative to 36B4. Values are mean ± SE. n=3 WT Sham, n=5 SIRT5KO Sham, n=4 WT TAC, n=7 SIRT5KO TAC. Males, 18-19 weeks of age at end of study. Two-way ANOVA with multiple comparisons, Bonferroni correction. * p ≤ 0.05; Effect of TAC if no significance in multiple comparisons tests. + p ≤ 0.05

Decreased cardiac energy production is a common feature that coincides with developing cardiac dysfunction. To determine if SIRT5KO mice had altered cardiac
energetics in response to TAC, we measured cardiac efficiency from the hemodynamic data using pressure volume area (PVA) analysis (Figure 9 F and G). Pressure volume area (PVA) is the sum of energy generated for mechanical work (stroke work) and energy that is generated but not used (potential work), whereas cardiac efficiency is defined as the ratio of stroke work to pressure volume area (stroke work + potential work). PVA in the SIRT5KO heart trended lower compared to the WT heart in both sham and TAC conditions (Figure 9 F), which was driven mainly by slightly lower stroke work in the SIRT5KO hearts compared to WT hearts (Table 2). We observed no differences in potential work across all four conditions. While we found no significant differences in cardiac efficiency (Figure 9 G), a subset of animals in the SIRT5KO TAC group appeared to have decreased cardiac efficiency compared to the other conditions tested (Figure 9 G), consistent with the idea that cardiac dysfunction develops at an accelerated rate in SIRT5KO mice. Together, these data show that after 4 weeks of TAC, both WT and SIRT5KO maintain energy generation compared to their sham controls. Overall the SIRT5KO mice have a trend of decreased energy production compared to the WT mice. These data suggest that despite decrements in systolic and diastolic function, both WT and surviving SIRT5KO mice have compensatory metabolic pathways for energy generation to maintain cardiac efficiency.
Figure 9: SIRT3KO mice have decreased ejection fraction and preserved cardiac energetics 4-weeks post-TAC

Pressure-volume loops with inferior vena cava occlusion were performed 4 weeks post TAC or sham surgery (A-D). (A) Representative images for wild-type sham, (B) SIRT5KO sham, (C) wild-type TAC, and (D) SIRT5KO TAC. Red trace indicates ESPVR (end systolic pressure volume relationship), green trace indicates EDPVR (end diastolic pressure volume relationship). (E) Ejection fraction. (F) Pressure volume area analysis. (G) Cardiac efficiency (stroke work/pressure volume area) (G). n=6 WT sham, n=9 SIRT5KO sham, n=7 WT TAC, n=11 SIRT5KO TAC. Combined results from 4 independent experiments. Males, 12-21 weeks of age at end of study. Two-way ANOVA with multiple comparisons, Bonferroni correction. * p \leq 0.05; Effect of TAC if no significance in multiple comparisons tests. + p \leq 0.05.

3.2.3 Protein succinylation is increased in SIRT5KO hearts and abundant on enzymes in oxidative metabolism

To determine if WT and SIRT5KO mice have similar metabolic compensatory mechanisms in response to TAC-induced cardiac stress, we combined proteomic and
metabolomic high-resolution mass spectrometry profiling in whole-tissue cardiac lysates. Because SIRT5 is a potent demalonylase, desuccinylase, and deglutarylase, we first measured the corresponding post-translational modifications by Western blotting (Figure 10 A-E). We found that lysine succinylation was significantly elevated across a wide range of proteins in whole-tissue lysates of SIRT5KO hearts under basal conditions (Figure 10 A). Other acyl-lysine modifications regulated by SIRT5 had a small (malonylation, Figure 10 B) or no (glutarylation, Figure 10 C) increases on proteins in SIRT5KO hearts under basal conditions, indicating that succinylation is the primary modification regulated by SIRT5 in the heart. Consistent with SIRT5 having weak deacetylase activity, we found no changes in protein acetylation in the SIRT5KO heart (Figure 10 D).

Metabolic stressors can lead to changes in protein acylation. For example, increases in lysine acetylation have been observed in both mouse models of heart failure (Karamanlidis et al., 2013; Martin et al., 2017) and in human patients in end-stage heart failure (Horton et al., 2016). Therefore, we profiled changes in cardiac protein acylation in WT and SIRT5KO mice in sham and TAC conditions. We observed TAC in WT mice leads to a slight reduction in global protein succinylation in whole-tissue lysates (Figure 10 F and G). Like WT mice, TAC in SIRT5KO mice also leads to a reduction in global protein succinylation (Figure 10 F and G), suggesting that shifts in succinyl-CoA metabolism, and therefore succinylation, are occurring in both WT and SIRT5KO mice
under TAC-induced cardiac stress. Western blot profiling showed no changes in malonylation, glutarylation, or acetylation with TAC (Western blots image quantification summarized in Figure 10 G; images not shown).

Since succinylation was the primary modification regulated by SIRT5 in the heart, and because basal conditions captured the largest breadth of protein succinylation, we performed succinyl-proteomics using label-free quantitative mass spectrometry of WT and SIRT5KO whole heart tissue. Cardiac tissue was homogenized, digested, and peptides containing succinyl-lysine modifications were enriched using an anti-succinyl-lysine affinity matrix. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to identify specific sites of lysine succinylation across the cardiac proteome, and then normalized to protein abundance. Overall, we identified 1448 unique proteins containing one or more sites of lysine succinylation (Figure 10 H). In total, 6124 unique peptides were identified, with the majority (93.8%) of peptides containing a single succinylated lysine. Most peptides identified displayed an increase in lysine succinylation upon SIRT5 ablation (Figure 10 I). We defined potential SIRT5 targets as sites of lysine succinylation that increased at least 2-fold with a p-value less than or equal to 0.05 (Figure 10 I, orange dots). Using this criterion, we identified 2,457 peptides that mapped to 766 unique proteins. Comparing the number of SIRT5 targets identified in our SIRT5KO cardiac succinyl-proteomics study to previously published similar studies (Boylston et al., 2015; Sadhukhan et al., 2016), we find that we
have greatly increased the number of identified sites of lysine succinylation with the potential to be regulated by SIRT5 (Figure 10 J). Next, we used Ingenuity Pathway Analysis (IPA) to determine pathways that were enriched in proteins containing lysine succinylation. The top pathways with succinylated proteins were primarily involved in oxidative metabolism, including oxidative phosphorylation, TCA cycle, BCAA degradation, and fatty acid beta-oxidation (Figure 10 K). The proteins identified in these pathways tended to have increased succinylation in the SIRT5KO compared to WT heart. While some instances of pathways with overall decreased succinylation were observed in the SIRT5KO heart, these pathways tended to represent minor or questionable metabolic pathways in the heart, such as gluconeogenesis (Doenst et al., 2013). We again compared the results of the pathway analysis from our dataset to the results of pathway analyses from previously published datasets (Boylston et al., 2015; Sadhukhan et al., 2016) (Figure 10 L). The three pathways that were common to all succinyl proteomics datasets were oxidative phosphorylation, TCA cycle, and fatty acid metabolism/oxidation. We found additional overlap between pathways enriched for succinylation in our dataset and the dataset generated by Boylston et al., which included BCAA degradation, glutaryl-CoA degradation, and ketogenesis. Together, these data confirm that sites of increased lysine succinylation in SIRT5KO mouse hearts occur on proteins involved in major oxidative metabolic pathways.
Figure 10: Succinylation is dramatically increased in the SIRT5KO heart and abundant on enzymes in oxidative metabolism
Western blots of (A) succinyl-lysine, (B) malonyl-lysine, (C) glutaryl-lysine, and (D) acetyl-lysine in WT and SIRT5KO mouse hearts at baseline. (E) Quantification of acyl-lysine blots. Acylation signal was normalized to total protein using BioRad stain free technology. Values are mean ± SE. n=3 WT, n=3 SIRT5KO. Student’s t-test. * p ≤ 0.05. Males, 8 weeks of age (panels A-E). (F) Western blot of succinyl-lysine in WT and SIRT5KO mouse hearts after 4 weeks of TAC. (G) Quantification of acyl-lysine blots. Acylation signal was normalized to total protein using BioRad stain free technology. Values are mean ± SE. n=3 WT sham, n=5 SIRT5KO sham, n=4 WT TAC, n=7 SIRT5KO TAC. Two-way ANOVA with multiple comparisons, Bonferroni correction. * p ≤ 0.05. Males, 18-19 weeks of age at end of study (panels F-G). (H) Summary of succinylated proteins and peptides identified via succinyl-proteomics. (I) Volcano plot of log_2 fold change in succinylation on peptides identified in SIRT5KO heart relative to wild-type heart and their associated –log_{10} p-values. (J) Comparison of number of SIRT5 targets identified in previous studies. (K) Ingenuity Pathway Analysis (IPA) summary of top pathways enriched with succinylated proteins. n=3 wild-type, n=3 SIRT5KO. Males, 11 weeks of age (panels H-I, K). (L) Venn diagram illustrating overlap of top pathways enriched with lysine succinylation in our study compared to previously published studies. Sadhukahn et al. used DAVID to perform pathway analysis, Boylston et al. used IPA to perform pathway analysis.

3.2.4 Oxidative metabolism is impaired in the SIRT5KO mouse 4-weeks post-TAC

Since pathways with a high number of proteins can be overrepresented in pathway analyses and artificially enhance p-values, we integrated the proteomics with unbiased metabolomic profiling in an effort to identify the primary metabolic lesions in SIRT5KO mouse hearts. Metabolite data collected from high-resolution mass spectrometry were uploaded to MetaboAnalyst 3.0 (Xia and Wishart, 2016; Xia et al., 2015), and analyzed for relevant groups of metabolites that were changing across conditions and genotypes. This analysis revealed multiple acyl-carnitines were decreased in the SIRT5KO TAC compared to SIRT5KO sham heart (Figure 11 A), while
few metabolites changed greater than 2-fold when comparing SIRT5KO sham vs. WT sham, SIRT5KO TAC vs. WT TAC, and WT TAC vs. WT sham (data not shown). Therefore, we extracted and analyzed the acyl carnitine data and found that overall these lipid metabolites were decreased after TAC in the SIRT5KO heart compared to the sham condition (Figure 11 B). Although acyl-carnitines trended towards decreased in the WT TAC compared to WT sham heart, the differences were not significant. Furthermore, we found long-chain fatty acids (palmitate and stearate) accumulated in both TAC conditions (Figure 11 C). However, the metabolites of the oxidation of these long chain fatty acids (C10:0, C12:0, and C14:0) are increased in the WT TAC condition, yet decreased in the SIRT5KO TAC condition compared to WT sham. Together, this metabolic signature suggests that long chain fatty acids are not metabolized to the same extent in the SIRT5KO TAC compared to WT TAC condition, and further suggest that fatty acid oxidation could be impaired in SIRT5KO TAC hearts.

To further interrogate these findings, we examined known mechanisms that regulate fatty acid oxidation. First, we performed RT-qPCR on key genes involved in fatty acid oxidation to determine if decreased acyl-carnitines in the SIRT5KO heart could be due to differences in transcriptional regulation of the fatty acid oxidation machinery. Indeed, reduced expression (Two Way ANOVA p ≤ 0.05; variation due to TAC) of Acadm, and Pppara in TAC hearts compared to sham hearts is consistent with reduced fatty acid oxidation in early stages of cardiac hypertrophy (Figure 11 D) (Akki et al.,
However, we found no significant differences between genotypes, suggesting that the significant decrease of acyl-carnitines observed in the SIRT5KO TAC heart compared to SIRT5KO sham heart was not due to differences in transcriptional regulation. In contrast, consistent with other sirtuin knock-out models, several enzymes in the fatty acid oxidation pathway were hyperacylated (Figure 11 E). Thus, fatty acid oxidation might be impaired in this model due to changes in protein acylation of the oxidation machinery directly. However, analyses of the metabolomic datasets revealed additional biochemical abnormalities in the SIRT5KO TAC heart compared to SIRT5KO sham heart.
Figure 11: Fatty acid oxidation is impaired in SIRT5KO TAC hearts

(A) Volcano plot of metabolites detected comparing SIRT5KO TAC to SIRT5KO sham. (B) Metabolite profiles of acyl-carnitines (* p ≤ 0.05 compared to SIRT5KO sham). (C) Metabolite profile of free fatty acids. (D) Cardiac mRNA expression of CD36, Cpt1, Acadvl, Acadl, Acadm, Ppara, Ppard, and Pgc1α relative to 36B4. Values are mean ± SE (panels B-D). n=3 WT Sham, n=5 SIRT5KO Sham, n=4 WT TAC, n=7 SIRT5KO TAC. Males, 18-19 weeks of age at end of study (panels A-D). Two-way ANOVA with multiple comparisons, Bonferroni correction. * p ≤ 0.05; Effect of TAC if no significance in multiple comparisons tests. + p ≤ 0.05. (E) Fold-change of all succinylated peptides identified on enzymes in fatty acid oxidation. Horizontal maroon bar represents the mean fold-change of all peptides identified on a single protein. n=3 wild-type, n=3 SIRT5KO. Males, 11 weeks of age.

Because glucose is the other main fuel metabolized by the heart, we inspected shifts in metabolites of glucose oxidation in the WT and SIRT5KO hearts. We found a trend toward increased glycolytic metabolites (Figure 12 A) and pentose phosphate pathway metabolites (Figure 12 B), suggesting an increase in glycolysis and pentose phosphate pathway flux. Importantly, the metabolic shifts observed here are consistent with several models of cardiac hypertrophy (Doenst et al., 2013).
Next, we sought to determine if a signature of elevated glucose metabolism corresponded with an increase in TCA cycle metabolites, as would be expected for complete glucose oxidation. However, TCA cycle metabolites downstream of pyruvate were generally reduced in response to TAC (Figure 12 C), which were further decreased in the SIRT5KO hearts; together, the directionality of these metabolic shifts suggest glucose-derived carbon is not entering the TCA cycle, and further suggests a mismatch between TCA cycle anaplerosis and cataplerosis. To further characterize regulation of carbon entry into the TCA cycle, we examined the phosphorylation of PDH since this is a primary control mechanism for pyruvate entry into the TCA cycle. Phosphorylation of PDH at Ser293 was increased in the SIRT5KO TAC heart compared to the SIRT5KO sham condition (Figure 12 D and E), a mark indicative of uncoupled glycolysis from glucose oxidation (Sankaralingam and Lopaschuk, 2015) via less PDH activity (Rardin et al., 2009). Supporting these findings, the lactate/pyruvate ratio in the SIRT5KO TAC heart compared to the SIRT5KO sham heart was elevated (Figure 12 F), suggesting glucose carbon is shunted from elevated glycolysis into lactate and not pyruvate.

We again interrogated known mechanisms of glycolytic and TCA cycle control. RT-qPCR of key genes involved in glucose metabolism show that Glut1 (the main glucose transporter in the hypertrophic heart) is modestly increased in the SIRT5KO TAC heart compared to the SIRT5KO sham condition (Figure 12 G); however, differences in transcription were subtle. Interestingly, succinyl proteomics indicated that
few enzymes in glycolysis were hypersuccinylated (Figure 12 H), while like other oxidative pathways, enzymes in the TCA cycle were hypersuccinylated in the SIRT5KO heart (Figure 12 I). Most of the glycolytic enzymes identified had an average log2 fold-change less than zero, which indicated overall hyposuccinylation of glycolytic proteins in SIRT5KO hearts compared to WT. Together, these data support the overall notion that hypersuccinylation occurs primarily on enzymes in mitochondrial oxidative pathways. Overall, our findings demonstrate that although flux through glycolysis appears elevated in both WT and SIRT5KO TAC hearts, complete glucose oxidation is reduced and TCA cycle carbon is lower in the SIRT5KO TAC heart.
Figure 12: Glucose oxidation is uncoupled from glycolysis in SIRT5KO TAC hearts

Metabolite profiles of (A) glycolytic metabolites, (B) pentose phosphate metabolites, and (C) TCA cycle metabolites. Values are mean ± SE. (D) Western blot of phospho-PDH and total PDH. (E) Quantification of blot, normalized to total protein, boxplots. (F) Ratio of lactate to pyruvate normalized to WT sham, boxplots. (G) Cardiac mRNA expression of Glut4, Glut1, Ldha, and Pdk4 relative to 36B4. Values are mean ± SE. n=3 WT Sham, n=5 SIRT5KO Sham, n=4 WT TAC, n=7 SIRT5KO TAC. Males, 18-19 weeks of age at end of study. Two-way ANOVA with multiple comparisons, Bonferroni correction. * p ≤ 0.05; Effect of TAC if no significance in multiple comparisons tests. + p ≤ 0.05 (panels A-G). Fold-change of all succinylated peptides identified on enzymes in (H) glycolysis, and (I) TCA cycle. Horizontal maroon bar represents the mean fold-change of all peptides identified on a single protein. n=3 wild-type, n=3 SIRT5KO. Males, 11 weeks of age (panels H-I).

Given that both decreases in fatty acid oxidation and TCA cycle metabolism are observed in SIRT5KO TAC hearts, we hypothesized that overall mitochondrial oxidative
metabolism might be impaired. To assess the mitochondrial redox state in WT and SIRT5KO TAC hearts, we extracted the beta-hydroxybutyrate/acetoacetate ratio (BOHB/Acac) from the metabolomic dataset, because it is a reliable indicator of the mitochondrial NAD\(^+\)/NADH ratio (Krebs et al., 1971). The SIRT5KO TAC mice have a strong trend of decreased BOHB/Acac (p=0.110) (Figure 14 A), indicative of increased mitochondrial NADH. Increased NADH could be due to lower NADH oxidation and OXPHOS activity. To assess the status of OXPHOS in the WT and SIRT5KO hearts, we first measured representative OXPHOS complex subunits by Western blotting and observed equal amounts of mitochondrial protein content between the WT and SIRT5KO hearts (Figure 14 B), suggesting that complex formation is normal. This is consistent with previously published findings in SIRT5KO livers (Zhang et al., 2017).

Interestingly, the SIRT5KO hearts appear to be in an energetically stressed state, as shown by AMPK phosphorylation (Figure 13). Therefore, we propose a model where that electron flow through OXPHOS is impaired in the absence of SIRT5. Consistent with hypersuccinylation of other metabolic pathways, we found that many protein subunits of OXPHOS contained multiple sites of lysine succinylation, with many having at least one site of succinylation with a fold change of greater than 20 in SIRT5KO hearts compared to WT (Figure 14 C). Further supporting this model, impairments in OXPHOS could explain a lower NAD\(^+\)/NADH; given the energetic observations and metabolite
profiles, we conclude that mitochondrial dehydrogenase activity is not responsible for the reductive mitochondrial environment.

Figure 13: Pressure overload induced cardiac hypertrophy leads to an energetic stress

(A) Western blot of phospho-ACC and total ACC. (B) Quantification of blot. Normalized to total protein. (C) Western blot of phospho-AMPK and total AMPK. (D) Quantification of blot. Normalized to total protein. Boxplots depict the interquartile range with whiskers plotted to the minimum and maximum values. The horizontal line within the box is the median value and the “+” is the mean value. (E) Nucleotides from whole heart tissue. Values are mean ± SE. (F) PCr/ATP ratio normalized to WT sham, boxplots. A-F: n=3 WT sham, n=5 SIRT5KO sham, n=4 WT TAC, n=7 SIRT5KO TAC. Two-way ANOVA with multiple comparisons, Bonferroni correction; * p ≤ 0.05. Effect of TAC if no significance in multiple comparisons tests; + p ≤ 0.05. Males, 18-19 weeks of age at end of study.
Overall, our data reveal metabolic abnormalities in fatty acid oxidation and TCA cycle function, which are coincident with protein hyperacylation in SIRT5KO hearts under chronic stress (Figure 14 D). These metabolic and biochemical deficiencies likely lead to an energetic crisis in the heart, which could explain the increased mortality in SIRT5KO mice with pressure overload.

Figure 14: Oxidative phosphorylation may be a primary lesion of impaired oxidative metabolism

(A) Ratio of beta-hydroxybutyrate to acetoacetate normalized to WT sham, boxplots. n=3 WT Sham, n=5 SIRT5KO Sham, n=4 WT TAC, n=7 SIRT5KO TAC. Males, 18-19 weeks of age at end of study. Two-way ANOVA with multiple comparisons, Bonferroni correction. (B) Western blot of OXPHOS cocktail antibody in WT and SIRT5KO heart tissue. (C) Fold-change of all succinylated peptides identified on enzymes in oxidative phosphorylation. Horizontal maroon bar represents the mean fold-change of all peptides identified on a single protein. n=3 wild-type, n=3 SIRT5KO.
Males, 11 weeks of age. (D) Working model. Cardiac hypertrophy is accompanied by shifts in metabolism including increased glycolysis and decreased fatty acid oxidation that ultimately fail to meet the energetic requirements of the heart (left). In the SIRT5KO heart (right), the conical shifts in metabolism are observed with hypertrophy, however there is an earlier reduction in oxidative metabolism. Succinylation of oxidative pathways may contribute to this phenotype. We suggest that impairment of OXPHOS leads to inhibition of glucose oxidation via phosphorylation of PDH and subsequent decreases in TCA cycle and fatty acid oxidation. Both pathways appear to be more decreased in the SIRT5KO hypertrophic heart compared to WT hypertrophic heart. This ultimately results in an energetic stress that leads to early death in the SIRT5KO mouse after TAC.

3.3 Discussion

The overall goal of this study was to define the major role of SIRT5 on cardiac metabolism by exposing whole-body SIRT5KO mice to the cardiac stress of chronic pressure overload and the subsequent cardiac hypertrophic response. Taken together, our data support a model where SIRT5 is required to maintain oxidative metabolism under chronic pressure overload; we discovered that ablation of SIRT5 results in significantly decreased survival upon TAC. In the absence of differences in cardiac function, we suggest that one major caveat of our study is survival bias, and suggest that reduced survival could be due to an accelerated progression impaired oxidative metabolism that leads to a bioenergetics stress. The mechanistic basis for these metabolic deficits could be explained by two discrete, but non-mutually exclusive models.

First, our data, consistent with historical data, show that hypersuccinylation occurs on key enzymes in oxidative pathways. As such, hyperacylation could inhibit
metabolic pathways that are required for energy generation under cardiac stress. Widespread hyperacylation across several enzymes and metabolic pathways could coordinately alter cardiac energy generation, thereby accelerating maladaptive alterations in response to pressure-overload-induced hypertrophy and ultimately heart failure. Supporting this model, we investigated the proteomic profile of oxidative pathways including fatty acid oxidation (Figure 11), the TCA cycle (Figure 12), and OXPHOS (Figure 14) and found that proteins in these pathways had multiple sites of lysine succinylation. Some previous examples show hypersuccinylation increases enzyme activity (Boylston et al., 2015; Park et al., 2013), while other studies show lysine acylation more often decreases enzyme activity (Chhoy et al., 2016) and impairs flux through metabolic pathways; together, hyperacylation could re-route carbon metabolism away from an oxidative, energy-producing state. Thus, in this model, multiple succinylation events across multiple proteins contribute to the metabolic and cardiac phenotypes.

Alternatively, our data also support a new model, in which a primary defect in OXPHOS in the SIRT5KO mouse heart leads to an elevation in mitochondrial NADH (reduced form). This proximal metabolic lesion in OXPHOS, likely by hypersuccinylation of multiple enzymes in OXPHOS and impaired electron flow through this pathway, coordinately alters mitochondrial oxidative metabolism (Figure 14 D). Supporting this model, SIRT5 was recently described to regulate oxidative
phosphorylation via its desuccinylase activity in liver (Zhang et al., 2017), which identified the electron transport chain as the top pathway targeted by SIRT5 and further found that complex II and ATP synthase activities were significantly decreased in mitochondria isolated from SIRT5KO mouse liver.

If these studies hold true in SIRT5KO hearts, impairment of OXPHOS in the SIRT5KO TAC heart could explain observations of metabolic derangements that occurred upstream of OXPHOS in this model. Specifically, a lower NAD⁺/NADH ratio (elevated NADH) activates pyruvate dehydrogenase kinase (PDK), which increases phosphorylation of PDH and inhibits its activity (Figure 12 D and E). Inhibition of PDH will lead to an increase in lactate formation (Figure 12 F), and a concomitant decrease in the TCA cycle carbon in the SIRT5KO TAC mouse. Furthermore, since the rate of fatty acid oxidation is dependent on the rate of supply of TCA carbon (Fritz, 1961), this model could also explain the decrease in fatty acid oxidation observed in the SIRT5KO TAC condition (Figure 11 B and C). Although NAD⁺/NADH is decreased in the SIRT5KO TAC heart and may impact the activity of other mitochondrial sirtuins, acetylation does not increase in the SIRT5KO TAC condition (Figure 10 G), suggesting that SIRT3 activity may not be affected. However, the accumulation of metabolic intermediates (i.e. acetyl-CoA or succinyl-CoA) are known to drive protein lysine acylation (Horton et al., 2016; Wagner et al., 2017) and acetyl-CoA trends towards decreased in the SIRT5KO TAC compared to WT TAC condition (Figure 12 C). This is consistent with decreased PDH
activity (Figure 12 D), and may explain the absence of increased acetylation even if SIRT3 is inactive.

Overall, this alternative model posits that a primary node of impaired oxidative metabolism could occur at OXPHOS, which ultimately causes an energetic crisis and explains the decreased survival in SIRT5KO mice with chronic pressure overload. Further supporting this concept, inborn errors of metabolism that affect the respiratory chain often cause decreased mitochondrial NAD+/NADH and increased glycolytic flux, which results in an increased lactate to pyruvate ratio and a clinical presentation of hypertrophic cardiomyopathy (Das and Illsinger, 2012). However, given the multifaceted roles of NAD⁺ (Hershberger et al., 2017b), a major challenge moving forward will be to disentangle these two competing models, and the contribution of lysine acylation/sirtuin activity and mitochondrial redox state to alterations in metabolism (Lee et al., 2016).

Regardless of the model, we would expect an accelerated trajectory of cardiac dysfunction in the setting of impaired oxidative metabolism, which often precedes overt heart failure (Sankaralingam and Lopaschuk, 2015). Shifts in metabolism are well characterized in models of pressure-overload-induced cardiac hypertrophy. Under normal conditions, most ATP (70-90%) produced in the heart is from the oxidation of fatty acids (Doenst et al., 2013). However, with development of hypertrophy, the contribution of fatty acids to cardiac ATP is decreased while increased rates of glycolysis
contribute more ATP than non-hypertrophied hearts (Allard et al., 1994; Kolwicz and Tian, 2011). Eventually, the metabolic adaptations are not sufficient to maintain cardiac energetics as oxidative metabolism continues to decline and cardiac dysfunction progresses. Consistent with previous observations, both WT and SIRT5KO hearts showed expected shifts in metabolism with TAC (increased glycolysis and decreased fatty acid oxidation), with evidence of accelerated defects in oxidative metabolism uniquely in the SIRT5KO TAC condition. Importantly, uncoupling of glycolysis from glucose oxidation as observed in the SIRT5KO TAC animals can cause decreased cardiac efficiency and is detrimental to heart function (Sankaralingam and Lopaschuk, 2015).

The most salient finding presented here is that upon chronic TAC-induced cardiac stress, SIRT5KO mice have significantly reduced survival compared to WT mice (Figure 6 A). The apparent discordance between the observed subtle functional defects and significant mortality may be explained by survivor (or survivorship) bias (Shermer, 2014). Over half of the SIRT5KO TAC mice had already died at the 4-week post-TAC time-point, making the surviving group of animals more homogeneous and the cause of future mortality more difficult to predict (Zhang and Pincus, 2016). Characterizing cardiac function at multiple early time points after the TAC procedure and/or stratifying mice based on cardiac function may be required to detect a marked defect in cardiac function in the SIRT5KO mice. Finally, it is possible that the phenotypes described here are due to cell non-autonomous roles of SIRT5 (i.e. cells or tissues beyond the
cardiomyocyte). Indeed, a role for SIRT3 in preventing fibrosis by activity in fibroblasts has recently been described (Sundaresan et al., 2015). A tissue-specific SIRT5KO mouse model will be required to determine the cardiomyocyte-intrinsic roles of SIRT5.

Together, our results suggest a model in which the SIRT5KO mouse has accelerated development of pressure-overload-induced cardiomyopathy (Figure 14 D). We predict that hypersuccinylation of oxidative pathways, especially OXPHOS, reduces energy production in SIRT5KO mouse hearts under TAC. However, survivor bias makes it difficult to uncover the molecular cause of mortality. More frequent monitoring of cardiac function may be required to more accurately define the mechanistic step(s) preceding mortality. Overall, this study points to an accelerated cardiac dysfunction in the SIRT5KO TAC animals as an explanation for the increased mortality observed with chronic TAC. Future studies on SIRT5 will identify how succinylation of mitochondrial proteins influences energy homeostasis in response to cardiac stress.

3.4 Summary

The goal of this part of the dissertation was to interrogate the physiological role of SIRT5 in the heart. Based on previous data, there is evidence that SIRT5 has a cardioprotective role in the heart, though there are no obvious cardiac phenotypes at baseline. Therefore, in collaboration with the Duke Cardiovascular Physiology Core, we used the stress of pressure overload via TAC to induce cardiac hypertrophy. Under this
condition we found that, compared to WT littermate controls, SIRT5KO mice have
decreased survival. Using the techniques of echocardiogram and pressure volume loop
hemodynamics, we found that there were no differences in cardiac morphology and
function 4-weeks post-TAC in the WT and SIRT5KO groups. We determined that
succinylation was the most dramatically changed modification in the heart in the
absence of SIRT5 and used a combined succinyl-proteomics and metabolomics approach
to look for any molecular signatures that could provide insight as to the mechanism of
decreased survival in SIRT5KO mice with TAC. We concluded that it was not the effect
of a specific enzyme, but likely the accumulation of succinylation on multiple enzymes
and pathways that contributed to the overall apparent decrease in oxidative metabolism.
Future studies using stable isotope tracing in a perfused hanging heart are required to
determine the overall contribution of substrates to ATP production under the TAC
condition. Our work lays the groundwork for pursuing this line of investigation as a
potential mechanism for increased mortality with TAC. Finally, our work was
conducted in a whole-body SIRT5KO mouse model, and we were curious if the
phenotypes observed in this model were due to an effect of SIRT5 in the heart, or a
whole-body effect of SIRT5. To begin to answer this question, we developed and
characterized a cardiomyocyte-specific SIRT5KO mouse model that was subjected to
pressure overload induced hypertrophy. This work is discussed in Chapter 4 of this
dissertation.
4. Heart-Specific SIRT5KO Mouse Studies

4.1 Introduction

Sirtuin 5 (SIRT5) is an NAD⁺-dependent demalonylase (Du et al., 2011; Peng et al., 2011), desuccinylase (Du et al., 2011), and deglutarylase (Tan et al., 2014) that resides primarily in mitochondria. Previous studies demonstrated that SIRT5 desuccinylase activity is important in maintaining cardiac function and metabolism in response to stress. In a model of ischemia-reperfusion injury, infarct size in SIRT5KO mouse hearts was greater than WT littermates (Boylston et al., 2015). Additionally, SIRT5 depletion was detrimental to cardiac function at 39-weeks of age, with defects in cardiac fatty acid oxidation (Sadhukhan et al., 2016). Finally, we recently published work describing increased mortality, and impaired oxidative metabolism in SIRT5KO mice compared to WT littermates with the stress of chronic pressure overload-induced cardiac hypertrophy (Hershberger et al., 2017a). Each of these three studies on the role of SIRT5 in the heart performed succinyl proteomic measurements in WT and SIRT5KO heart tissue in order to identify enzymes and pathways potentially regulated by SIRT5. In all three studies, key pathways in mitochondrial metabolism including oxidative phosphorylation, TCA cycle, and fatty acid oxidation were identified as regulated by SIRT5 given the increase in succinylation on many enzymes in these pathways with SIRT5 depletion (Hershberger et al., 2017a). Together, these data suggest that SIRT5 is important in maintaining cardiac function with oxidative metabolism as a key node of
regulation via SIRT5 desuccinylase activity. While these studies suggest that SIRT5 has a cardioprotective role, it remains unknown if the effect of SIRT5 is cardiomyocyte specific or due to a whole-body effect of SIRT5.

Most characterization of the physiological roles of mitochondrial sirtuins have been conducted in whole-body sirtuin KO mice. However, differing degrees of acetylation in SIRT3KO tissues (Dittenhafer-Reed et al., 2015) and malonylation and succinylation in SIRT5KO tissues (Nishida et al., 2015) suggests that the roles of sirtuins may differ between tissues. Recently, there has been more interest in understanding the tissue-specific effects of mitochondrial sirtuins in order to determine contribution of individual tissues to phenotypes described in germline depletion models. For example, whole body SIRT3KO mice on a high fat diet have accelerated development of obesity, insulin resistance, hyperlipidemia, and steatohepatitis (Hirschey et al., 2011). However, in a liver or skeletal muscle-specific (two tissues with the greatest influence on whole body metabolism) SIRT3KO model, there were no metabolic differences when compared to WT controls despite similar hyperacetylation profiles to the whole-body SIRT3KO model (Fernandez-Marcos et al., 2012). The authors of this study concluded that either a different tissue or a coordinated tissue response was responsible for the phenotypes observed in the whole-body SIRT3KO mouse. Another major phenotype in the whole-body SIRT3KO mouse is the development of spontaneous cardiac hypertrophy (Hafner et al., 2010; Pillai et al., 2010). It has recently been suggested that this phenotype is
recapitulated in a skeletal muscle and heart-specific SIRT3KO model (Martin et al., 2017). However, it is not yet known if this is exclusively a cardiomyocyte effect of SIRT3, as SIRT3 has also been shown to play a specific role in fibrosis (Sundaresan et al., 2015), a key component in the development of cardiac hypertrophy. Thus far, no studies have been published on the tissue-specific roles of SIRT4 or SIRT5. Thus, there is a need to develop tissue-specific sirtuin KO models in order to better understand the tissues contributing to the phenotypes revealed in whole-body KO models. To this end, we set out to determine if the phenotypes described in the whole-body SIRT5KO mouse under chronic pressure overload induced hypertrophy were recapitulated in a heart-specific SIRT5KO mouse model.

A well-established experimental tool to knockout genes in an inducible and cardiomyocyte-specific manner is the α-MHC-MerCreMer mouse model. In this model, two mutated estrogen receptors (Mer) flank a Cre transgene located upstream of the cardiomyocyte-specific myosin heavy chain-alpha (α-MHC) promoter (Sohal et al., 2001). This system requires delivery of tamoxifen to bind to the mutated estrogen receptors, translocate to the nucleus, and induce expression of Cre. One benefit of using an inducible system is that the gene can be depleted postnatally, circumventing problems with embryonic lethally or compensation during early development. However, a major caveat of this mouse model is established Cre toxicity in the heart, as evidenced by heart failure with high amounts of tamoxifen (Bersell et al., 2013), and
transient inflammation and hypertrophy with lower doses of tamoxifen (Lexow et al., 2013). Here, we present data on the development and characterization of an inducible cardiomyocyte-specific SIRT5KO mouse model. Further, we analyze the cardiac morphological and functional changes with chronic TAC, and survey the succinylation profile in this novel mouse model.

4.2 Results

4.2.1 Characterization of heart specific, tamoxifen inducible SIRT5KO mouse model

To test if the cardiac phenotypes observed in the whole body SIRT5KO mouse were heart intrinsic, we developed a heart-specific, tamoxifen-inducible, SIRT5KO mouse model. To generate this mouse, we crossed SIRT5\(^{fl/fl}\) (Yu et al., 2013) mice with \(\alpha\)MHC-MerCreMer mice to generate littermates with the following genotypes: SIRT5\(^{fl/fl}\); \(\alpha\)MHC-MerCreMer\(^{-/-}\) (hereto referred to as \(fl/fl\)) and SIRT5\(^{fl/fl}\); \(\alpha\)MHC-MerCreMer\(^{+/-}\) (hereto referred to as \(fl/fl;MCM\)). Additionally, the floxed alleles were crossed out of this line to generate a second line of \(\alpha\)MHC-MerCreMer\(^{-/-}\) and \(\alpha\)MHC-MerCreMer\(^{+/-}\) mice to generate \(\alpha\)MHC-MerCreMer\(^{+/-}\) (hereto referred to as MCM) mice to control for Cre toxicity.

Two methods of delivering tamoxifen were tested to determine the best way to achieve SIRT5 depletion in the heart. First, adult mice were fed a commercially available tamoxifen citrate diet (Envigo Tekland Diets, TD.130860) for 3 weeks. In the second
method, tamoxifen was dissolved in corn oil and given to adult mice by oral gavage at a dose of 80mg/kg for three consecutive days. We found that with either method of delivery, depletion of SIRT5 took more than two weeks (Figure 15). Feeding tamoxifen citrate for three weeks followed by two weeks of regular chow resulted in approximately 75% depletion of SIRT5 (Figure 15 A-B) while two weeks after the oral gavage regimen, there was about 50% depletion of SIRT5 (Figure 15 D-E). Since expression of Cre in the heart has been shown to be toxic and results in an acute cardiomyopathy (Lexow et al., 2013), we measured cardiac hypertrophy by calculating the ratio of heart weight to body weight at multiple time points throughout these pilot studies. Although there was only a small sample size at each time point (n=1 or 2), there were trends of increased hypertrophy in the fl/fl;MCM mice compared to the fl/fl mice after three weeks of the tamoxifen diet (Figure 15 C). This hypertrophy appeared to normalize after two weeks of regular chow following the tamoxifen diet. No differences in heart weight to body weight were observed between the fl/fl controls and fl/fl;MCM mice in the oral gavage pilot (Figure 15 F). Finally, we used RT-qPCR to further measure transcript levels of Sirt5 and markers of Cre toxicity including Nppa (Anf), Nppb (Bnp), and Il6. Although SIRT5 protein levels were not diminished until weeks after tamoxifen dosing, SIRT5 transcript levels were depleted at earlier time points (Figure 15 G). Maximal depletion of SIRT5 transcript levels occurred after two weeks of the tamoxifen diet (Figure 15 G, left panel) and one day after the oral gavage dosing regimen (Figure
Transcript levels of Cre toxicity markers were highest after three weeks of tamoxifen diet feeding or one day to one week post oral gavage dosing (Figure 15 H-J). In the tamoxifen diet pilot group, transcript levels of Anf, Bnp, and Il6 returned to control levels after two weeks of regular chow feeding. Anf and Bnp remained elevated in the oral gavage pilot group two weeks post oral gavage dosing (Figure 15 H and I). Based on these data, we proceeded with optimization of the tamoxifen diet feeding regimen because greater depletion of SIRT5 was achieved and we predicted that a shorter time on the diet would be sufficient to achieve depletion of SIRT5 and would result in less Cre toxicity. In studies described below, tamoxifen diet was fed for 8-10 days and we observed decreased evidence of Cre toxicity (data not shown).
Figure 15: Characterization of tamoxifen-inducible, heart specific SIRT5KO mouse model

Tamoxifen diet pilot: (A) Western blot of SIRT5 over time, (B) quantification of blot, (C) heart weight to body weight ratio. Tamoxifen oral gavage pilot: (D) Western blot of SIRT5 over time, (E) quantification of blot, (F) heart weight to body weight ratio. Blots were normalized to total protein using BioRad stain free technology. Transcript levels of (G) Sirt5 (H) Anf (I) Bnp and (J) Il6 normalized to 36B4 over time in tamoxifen diet (left) and tamoxifen oral gavage (right) pilots. Adult males were used with n=1-2 per group.

4.2.2 Protein succinylation accumulates over time

Given that SIRT5 is a protein lysine desuccinylase and that succinylation in the whole-body SIRT5KO heart is abundant, we looked at succinylation over time after
tamoxifen-induced ablation of Sirt5. Adult female mice were fed a tamoxifen citrate diet for 10 days followed by a regular chow diet. Mice were sacrificed immediately after the tamoxifen diet regimen, and 3-, 6-, 12.5-, and 32.5-weeks after the tamoxifen diet regimen. Western blotting was used to analyze succinylation and SIRT5 protein expression in whole heart lysates. Interestingly, we found that it took several weeks for protein succinylation to accumulate to levels that were comparable to succinylation in the whole-body SIRT5KO mouse (Figure 16 A and B). Specifically, 32.5 weeks after depletion of Sirt5, succinylation in fl/fl;MCM mouse hearts was about 3-fold greater than succinylation in the fl/fl mouse hearts. Although this was the maximum succinylation achieved in this time course, the whole-body SIRT5KO mouse heart has an approximate 5.5-fold increase in succinylation over the WT control. Thus, it is possible that sites of lysine succinylation would continue to accumulate if the time course was taken past 32.5 weeks. Interestingly, SIRT5 protein appeared to be maximally depleted 3 weeks after the tamoxifen diet feeding regimen (Figure 16 C). Together, this data shows that in our heart-specific inducible SIRT5KO mouse model, SIRT5 is depleted after 3 weeks of Sirt5 depletion, yet succinylation continues to increase for at least 30 weeks. This important finding provides a unique tool to assess the effects of SIRT5 ablation with varying degrees of protein lysine hypersuccinylation, which might allow us to disentangle direct effects of SIRT5 from indirect effects of protein succinylation.
Figure 16: Succinylation accumulates over time

(A) Western blot of protein succinylation and SIRT5 weeks after feeding of tamoxifen diet. Quantification of (B) succinylation and (C) SIRT5. Blots were normalized to total protein using Bio-Rad stain free technology. Adult females were used in this study with n=1-3 per group.

4.2.3 Effect of protein succinylation on cardiac function

Previous studies found no phenotype in the whole-body SIRT5KO mouse at baseline compared to WT controls (Hershberger et al., 2017a; Yu et al., 2013). However,
it is possible that the whole-body SIRT5KO mouse makes adaptations to compensate for the germline ablation of *Sirt5*. We used the heart-specific inducible model to determine if there was a difference in cardiac function at baseline with depletion of SIRT5. Additionally, we sought to determine if any phenotypes observed were an effect of SIRT5 ablation or accumulation of succinylation. In order to test this hypothesis, we generated two groups (early tamoxifen diet and late tamoxifen diet) with fl/fl;MCM experimental mice and fl/fl and MCM controls. The early tamoxifen diet (TD) group was fed tamoxifen for 8 days immediately after weaning, followed by a regular chow diet until the group was 22 weeks of age (Figure 17 A, top). The late tamoxifen diet group was fed regular chow for the first 17 weeks of age, then fed a tamoxifen diet for 8 days (Figure 17 A, bottom). This group was fed regular chow for 3.5 weeks before analysis of cardiac function to allow for normalization of cardiac function that might have been disrupted due to Cre toxicity. LV weight to body weight ratio was calculated to determine if there was an effect of Cre toxicity at the time of pressure volume loop analysis (Figure 17 B). All LV weight to body weight ratios appeared similar, though there was a strong trend of increased LV weight to body weight in the fl/fl;MCM (Early TD) group compared to fl/fl (Early TD) group. This is likely not due to Cre toxicity because the group had not been exposed to the tamoxifen diet for 16 weeks and is more likely due to one animal in the fl/fl;MCM (Early TD) group that is pulling the average.
LV weight to body weight ratio up. This data suggests that there was not an effect of Cre toxicity when pressure-volume measurements were taken.

Figure 17: Effect of SIRT5 versus succinylation on baseline cardiac function

Tamoxifen diet feeding regimen for Early TD group (A, top) and Late TD group (A, bottom). (B) Left ventricle weight to body weight ratio at end of study. Two-way ANOVA, Bonferroni correction for multiple comparisons. (C) Western blot of succinylation and SIRT5 in fl/fl;MCM mice from Early TD group and Late TD group. Quantification of (D) succinylation and (E) SIRT5. Blots were normalized to total protein using BioRad stain free technology. All mice used in this study were males. Early TD group: n=3 MCM, n=8 fl/fl, n=6 fl/fl;MCM. Late TD group: n=4 MCM, n=7 fl/fl, n=6 fl/fl;MCM.
Pressure-volume loops were conducted in both the late TD and early TD groups at 22 weeks of age. There were no differences between the fl/fl and fl/fl;MCM groups in either of the early TD or late TD groups (data not shown). This suggests that depletion of SIRT5 post development does not reveal cardiac phenotypes that were masked in the whole body SIRT5KO mouse. Additionally, there were no significant differences between the fl/fl;MCM (Late TD) group and fl/fl;MCM (Early TD) group for any of the measurements taken (Table 4 and Table 5). However, there were a few interesting trends of differences in systolic function between these two groups. Although baseline hemodynamic systolic function parameters including stroke volume, cardiac output, and stroke work (Table 4) trended higher in the fl/fl;MCM (Early TD) group compared to fl/fl;MCM (Late TD) group, these same trends were observed in the MCM arm, suggesting an effect of Cre instead of hypersuccinylation. In contrast, the trends of decreased contractility (ESPVR (linear) slope, ESPVR (Quadratic) E’max, and Emax; Table 5) in the fl/fl;MCM (Early TD) compared to fl/fl;MCM (Late TD) were not observed in the MCM arm, suggesting that an effect of hypersuccinylation in the absence of SIRT5 may be decreased contractility. Despite this finding, there remain multiple caveats in this system that warrant further optimization and testing.

First, although there was an increase in protein succinylation in the fl/fl;MCM (Early TD) group, it was only about 1.8 fold greater than the fl/fl;MCM (Late TD) group (Figure 17 C and D). More time between feeding the tamoxifen diet and conducting the
pressure volume loops would allow for the accumulation of more sites of lysine succinylation (Figure 16 A) and could potentially reveal a less subtle phenotype of decreased contractility with hypersuccinylation. Additionally, the depletion of SIRT5 is greater in the fl/fl;MCM (Early TD) group (~85%) compared to the fl/fl;MCM (Late TD) group (~60%) (Figure 17 C and E). Since the amount of SIRT5 protein between the groups differ, it is not possible to completely disentangle the effect of SIRT5 versus the effect of succinylation. Optimization of the length of time to reduce SIRT5 proteins to more similar levels will be important to best make use of this model.

**Table 4: Baseline Hemodynamic Parameters (Basal Succinylation versus Hypersuccinylation)**

Statistical comparisons were made using a Two-Way ANOVA with Bonferroni correction for multiple comparisons. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>fl/fl;MCM (Late TD) Basal Succinylation</th>
<th>fl/fl;MCM (Early TD) Hypersuccinylation</th>
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<tr>
<td>Heart Rate (BPM)</td>
<td>510.3 ± 17.3</td>
<td>503.7 ± 14.1</td>
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<tr>
<td>LVESP (mm Hg)</td>
<td>120.9 ± 7.6</td>
<td>119.4 ± 8.1</td>
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<tr>
<td>LVEDP (mm Hg)</td>
<td>3.80 ± 1.01</td>
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**Systolic Function Parameters**

<table>
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<th>fl/fl;MCM (Late TD) Basal Succinylation</th>
<th>fl/fl;MCM (Early TD) Hypersuccinylation</th>
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<tr>
<td>Stroke Volume (µl)</td>
<td>19.08 ± 3.12</td>
<td>22.47 ± 2.91</td>
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<td>Ejection Fraction (%)</td>
<td>52.30 ± 6.00</td>
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<td>Cardiac Output (µl/min)</td>
<td>9949 ± 1805.6</td>
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<td>Stroke Work (mmHg*µl)</td>
<td>1671 ± 377.4</td>
<td>2283 ± 337.5</td>
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<td>dP/dt_max (mm Hg/s⁻¹)</td>
<td>9428 ± 656.9</td>
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**Diastolic Function Parameters**

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<td>dP/dt_max (mm Hg/s⁻¹)</td>
<td>-8617 ± 549.8</td>
<td>-8801 ± 359.68</td>
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<tr>
<td>Tau (ms) (Weiss)</td>
<td>6.46 ± 0.26</td>
<td>5.97 ± 0.30</td>
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<tr>
<td>Tau (ms) (Glantz)</td>
<td>10.70 ± 0.35</td>
<td>10.34 ± 0.44</td>
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</table>
Table 5: Load Independent Hemodynamic Measures (Basal Succinylation versus Hypersuccinylation)

Parameters of LV compliance and LV contractility. Statistical comparisons were made using a Two-Way ANOVA with Bonferroni correction for multiple comparisons. Data are presented as mean ± SEM. EDPVR=End Diastolic Pressure Volume Relationship; ESPVR=End Systolic Pressure Volume Relationship; a=coefficient of curvilinearity; Vo=Volume intercept; E’max=maximum slope of quadratic ESPVR; PRSW=Preload Recruitable Stroke Work; Emax=maximal elastance

<table>
<thead>
<tr>
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<th>fl/fl;MCM (Late TD) Basal Succinylation</th>
<th>fl/fl;MCM (Early TD) Hypersuccinylation</th>
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<tr>
<td></td>
<td>n=6</td>
<td>n=6</td>
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<tr>
<td>Compliance Parameters</td>
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<tr>
<td>EDPVR (Linear) Slope</td>
<td>0.26 ± 0.08</td>
<td>0.23 ± 0.03</td>
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<tr>
<td>EDPVR (Linear) Intercept</td>
<td>-0.96 ± 0.65</td>
<td>-1.66 ± 1.02</td>
</tr>
<tr>
<td>EDPVR (Quadratic) b coefficient</td>
<td>0.080 ± 0.018</td>
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<td>EDPVR (Quadratic) a coefficient</td>
<td>0.782 ± 0.137</td>
<td>0.836 ± 0.140</td>
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<td>Contractility Parameters</td>
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<tr>
<td>ESPVR (Linear) Slope</td>
<td>7.74 ± 1.44</td>
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<tr>
<td>ESPVR (Linear) Intercept</td>
<td>-2.04 ± 1.42</td>
<td>-3.18 ± 4.23</td>
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<td>ESPVR (Quadratic) a</td>
<td>-0.58 ± 0.21</td>
<td>-0.39 ± 0.26</td>
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<td>ESPVR (Quadratic) V₀</td>
<td>1.94 ± 0.91</td>
<td>1.58 ± 3.18</td>
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<td>ESPVR (Quadratic) E’max</td>
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<td>PRSW Slope</td>
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<td>Emax</td>
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4.2.4 TAC in heart-specific SIRT5KO mice does not recapitulate the phenotype in whole-body SIRT5KO mice

Given that we didn’t observe any obvious differences in cardiac function between the fl/fl and fl/fl;MCM groups under baseline conditions, we next induced left ventricle cardiac hypertrophy by performing TAC, as we did previously in whole body
SIRT5KO mice (Hershberger et al., 2017a) and discussed in Chapter 3 of this dissertation. The most striking phenotype in whole-body SIRT5KO mice was decreased survival over the course of 16 weeks of TAC. We repeated this study in the fl/fl and fl/fl;MCM mice and did not observe a difference in the survival between the two groups with TAC (Figure 18 A). Within the first week, there was overall 30% mortality which was likely due to surgery (~20% mortality is expected with TAC). Over the remaining 15 weeks of observation, there was only one instance of mortality in each group. At the end of 16 weeks, gradients were measured on surviving mice and both groups had gradients well above the accepted 20 mmHg cut-off (Figure 18 D). Serial echocardiography was performed pre-TAC and 2-, 4-, 8-, 12-, and 16-weeks post-TAC. Wall thickness increased in fl/fl and fl/fl;MCM groups within the first two weeks of TAC (Figure 18 B), indicative of the development of hypertrophy. However, there was no difference between genotypes though, the fl/fl group appears to have increased wall thickness compared to the fl/fl;MCM group. LV weight to body weight was measured at the end of the study (Figure 18 E). In concordance with the echocardiography data, the fl/fl group had a trend of increased cardiac hypertrophy compared to the fl/fl;MCM group. Additionally, we observe a trend of reduced fractional shortening in both groups over 16 weeks of TAC with no differences between genotype (Figure 18 C). Together, these data show that the phenotype observed in the whole body SIRT5KO mouse of increased mortality
compared to WT controls with TAC does not repeat in the heart specific SIRT5KO mouse model under these conditions.

Figure 18: Heart specific SIRT5KO response to pressure overload induced cardiac hypertrophy

Males 4-5 weeks of age were fed tamoxifen citrate for 8 days followed by 14.5 weeks of regular chow. Mice were 20-21 weeks of age when TAC surgery was performed. Mice were followed for 16 weeks post TAC. (A) percent survival. Serial echocardiography was performed at 0-, 2-, 4-, 8-, 12-, and 16-weeks post TAC. (B) Wall thickness, and (C) Fractional shortening. At the end of study (D) gradient was determined. (E) Left ventricle weight to body weight ratio at end of study. n=7-10 fl/fl; n=5-10 fl/fl;MCM.
In the whole-body SIRT5KO heart, succinylation decreased in the 4-week post-TAC compared to pre-TAC SIRT5KO animals (Figure 10). We examined protein succinylation in pre-TAC and 16-week post-TAC fl/fl;MCM animals compared to fl/fl controls to determine if this repeated in the heart-specific SIRT5KO model. We found that protein succinylation was 2-fold higher in fl/fl;MCM mice compared to fl/fl mice in the pre-TAC group, and that protein succinylation was 4-fold higher in fl/fl;MCM mice compared to fl/fl mice in the post-TAC group (Figure 19 A and B). Additionally, there was an increase in protein succinylation when comparing the fl/fl;MCM 16-week post-TAC group and fl/fl;MCM pre-TAC group (Figure 19 A and B). In contrast to whole-body SIRT5KO mice where protein succinylation decreases with 4 weeks of TAC, we find that succinylation increases with 16 weeks of TAC in heart-specific SIRT5KO mice. In both models, there is no change in succinylation with TAC in the controls groups (WT or fl/fl). SIRT5 protein expression was the same in the fl/fl;MCM pre-TAC and 16-week TAC groups (Figure 19 A and C). Thus, there is a difference in protein succinylation in the whole-body SIRT5KO mouse model and heart-specific SIRT5KO mouse model both at the time of TAC surgery and in response to TAC although Sirt5 is ablated in the heart in both models. Together, this data suggests that the phenotype observed in the whole-body SIRT5KO mouse model with TAC may not be a direct effect of SIRT5 in the heart, but an effect of protein succinylation.
Figure 19: Succinylation increases over the course of 16-week TAC in fl/fl;MCM mice

Males 4-5 weeks of age were fed tamoxifen citrate for 8 days followed by 17.5 weeks of regular chow and were sacrificed (Pre-TAC group; n=3 fl/fl, n=3 fl/fl;MCM). Males 4-5 weeks of age were fed tamoxifen citrate for 8 days followed by 14.5 weeks of regular chow and underwent TAC or sham surgery for 16 weeks and were sacrificed (16 Week TAC group; n=7 fl/fl, n=5 fl/fl;MCM). (A) Western blot of succinylation and SIRT5 pre-TAC and post TAC. Quantification of (B) succinylation and (C) SIRT5. Blots were normalized to total protein using BioRad stain free technology.

4.2.5 Heart-specific SIRT5KO proteomics

To further explore this possibility, we performed succinyl-proteomics in the fl/fl and fl/fl;MCM mice at 15 weeks and 31 weeks post-tamoxifen diet feeding. Immediately after weaning, fl/fl and fl/fl;MCM mice were fed tamoxifen for 8 days and then returned to a regular chow diet. One group of mice were sacrificed 15 weeks after tamoxifen-induced ablation of Sirt5 (n=2 fl/fl and n=3 fl/fl;MCM) and a second group of mice were sacrificed 31 weeks after tamoxifen-induced ablation of Sirt5 (n=2 fl/fl and n=2
fl/fl;MCM). Left ventricle hearts were pulverized and processed at the same time for succinyl proteomics analysis using TMT 10plex Isobaric Labeling (ThermoFisher Scientific).

The goal of this study was to determine how the protein succinylation landscape changes over time after SIRT5 depletion. To begin to answer this question, we performed pathway analyses on a group of peptides that had increased succinylation compared to their respective time control (KO/WT at 15 weeks and KO/WT at 31 weeks; Figure 20 A and B) and compared to their genotype control (KO 31 weeks/KO 15 weeks and WT 31 weeks/WT 15 weeks; Figure 20 C). We used parameters of Log2 FC ≥1 and an adjusted p-value ≤ 0.1 (Benjamini-Hochberg correction; 10% FDR) to make a list of proteins for each given comparison that had increased succinylation. These lists were uploaded to PANTHER to perform statistical overrepresentation tests (Mi et al., 2013) to determine pathways that were significantly overrepresented. The top three significantly overrepresented pathways in each comparison (except WT 31 weeks/WT 15 weeks) were fatty acid beta-oxidation, tricarboxylic acid cycle, and oxidative phosphorylation or cellular amino acid catabolic process. Together, this data suggests that proteins in the same metabolic pathways are succinylated in the SIRT5KO heart at 15 weeks and 31 weeks-post SIRT5 depletion. There were no peptides with changes in succinylation when comparing the succinylation profile of WT hearts at 15 and 31 weeks after
tamoxifen feeding (data not shown), demonstrating that under basal conditions, there is little change in the sites of protein succinylation.

We hypothesized that the increase in succinylation that occurs in the SIRT5KO heart between 15 and 31 weeks post SIRT5 depletion was due to either an increase in the number of unique sites of succinylation, or a further increase in succinylation at lysine residues already succinylated. The succinyl proteomics performed here does not allow us to fully address the former possibility in that the absence of identification of a specific site of lysine succinylation may be due to a lack of coverage and not the absolute absence of succinylation. To explore the latter possibility, we plotted the fold change of all sites of lysine succinylation at 31 weeks (KO/WT) and 15 weeks (KO/WT) in fatty acid oxidation (Figure 20 D), the TCA cycle (Figure 20 E), and OXPHOS (Figure 20 F). We found that the fold change of at least 50% of succinylation sites on proteins in fatty acid oxidation and the TCA cycle increased from 15 weeks (light red circles) to 31 weeks (dark red circles) post SIRT5 depletion. Overall, there were fewer sites of succinylation that increased between these time points on proteins in OXPHOS. Notably, a number of sites that were succinylated greater than 2-fold at the 15-week time-point maintained the same fold change succinylation at 31 weeks. Only a few SIRT5 succinylation targets have been validated in the heart: lysine 179 and 335 on SDHA (Boylston et al., 2015), and lysine 351 on HDHA (Sadhukhan et al., 2016). When we compare the fold change of these sites at 15 weeks, 31 weeks, and the whole-body SIRT5KO (Figure 20 G-I), we find
that the log2 fold change is about the same in the 31-week and whole-body conditions, but trends lower in the 15-week condition. These data suggest that succinylation of SIRT5 targets may not occur immediately after depletion of SIRT5; indeed, these data suggest that maximal succinylation on validated targets of SIRT5 occurs 31 weeks after Sirt5 ablation.

Together, our data reveal that deletion of Sirt5 post-natally in the heart results in accumulation of protein lysine succinylation over the course of months, while SIRT5 protein expression is maximally depleted about 3 weeks after Sirt5 deletion by Cre. These findings provide a novel model to study the effect of loss of SIRT5 under conditions of basal succinylation and/or hypersuccinylation. Finally, the data presented in this study suggest that increased mortality with TAC in whole-body SIRT5KO mice is due to a non-cardiac intrinsic effect of SIRT5, or an effect of SIRT5 (or indirect effect of succinylation) in the heart that is only manifested with germline ablation of Sirt5.
Figure 20: Succinyl proteomics in heart-specific SIRT5KO mouse model over time

Volcano plots of (A) Log2 FC (KO/WT) 15 weeks, (B) Log2 FC (KO/WT) 31 Weeks, and (C) Log2 FC (31W/15W) KO. Log2 FC values are plotted against Benjamini-Hochberg corrected p-values with a 10% FDR. Text indicates the number of peptides and proteins in each group that have decreased succinylation (upper left) and increased succinylation (upper right). All sites of lysine succinylation identified in (E) fatty acid
oxidation, (F) the TCA cycle, and (G) OXPHOS. Dark red circles represent the Log2 FC (KO/WT) 31 weeks-post SIRT5 depletion and light red circles represent the Log2 FC (KO/WT) 15 weeks-post SIRT5 depletion. Sites of lysine succinylation are aligned vertically. Comparison of Log2 FC (KO/WT) of (H) SDHA:K179, (I) SDHA:K335, and (J) HADHA:K351 in 15 week, 31 week, and whole-body SIRT5KO hearts. P-values are adjusted for multiple comparisons within the same experiment. Data for the whole-body was published in (Hershberger et al., 2017a) and discussed in Chapter 3 of this dissertation.

4.3 Discussion

The major goal of this study was to determine if increased mortality in whole-body SIRT5KO mice exposed to pressure overload induced cardiac hypertrophy was a cardiac-intrinsic or whole-body effect of SIRT5. Exposure to 16-week TAC showed that the whole-body SIRT5KO model and the heart-specific SIRT5KO model behaved differently. This could be interpreted a few different ways. First, the increased mortality with chronic TAC in the whole-body SIRT5KO model could be due to a multi-tissue effect of SIRT5. Second, the phenotype observed in the whole-body SIRT5KO model could be an indirect effect of protein succinylation, and not a direct effect of SIRT5. Insight into the time course of succinylation that this model uncovered reveals a number of important questions that warrant further exploration.

First, we developed a novel, tamoxifen-inducible, cardiomyocyte specific SIRT5KO mouse model. To our knowledge, previous studies involving tissue-specific sirtuin KO mouse models have not been inducible, but driven by constitutive expression of Cre. A study characterizing a liver specific SIRT3KO mouse model and a skeletal
muscle specific SIRT3KO mouse model reported similar acetylation profiles compared to the whole body SIRT3KO mouse model (Fernandez-Marcos et al., 2012). In contrast, in the heart specific SIRT5KO mouse model, we find that succinylation accumulates over the time course of months after Sirt5 ablation, with no change in succinylation in fl/fl controls after feeding the tamoxifen diet (Figure 16). One emerging model suggests that acylation is a form of carbon stress and that sirtuins function to remove acyl-lysine modifications as a part of the protein quality-control network (Wagner and Hirschey, 2014). It is thought that acylation occurs non-enzymatically, and recent studies from our lab demonstrate that succinyl-CoA undergoes intramolecular catalysis to form a highly reactive cyclic anhydride intermediate (Wagner et al., 2017). In support of this notion, incubating cell protein lysates ex vivo with increasing concentrations of acyl-CoA’s increases the acyl-lysine signal as measured by Western blot (Wagner and Payne, 2013). We suggest that accumulation of succinylation in our model is a result of availability of highly reactive succinyl-CoA coupled with the loss of SIRT5, the only known lysine desuccinylase. Given the highly reactive intermediate that forms during intramolecular catalysis of succinyl-CoA and the great length of time to see any observable differences in lysine succinylation after SIRT5 depletion, we predict that the succinyl-CoA available for protein lysine succinylation is relatively low in abundance under basal conditions. Previous studies have shown that acetylation accumulates under disease conditions such as heart failure (Horton et al., 2016), when acetyl-CoA homeostasis is altered. To
further test this model of succinylation in the heart specific SIRT5KO mouse model, it would be of interest to increase succinyl-CoA concentrations and determine the effect on the time of increases in protein lysine succinylation.

Heart specific SIRT5KO mice were exposed to chronic pressure overload via TAC surgery for 16 weeks in order to determine if this mouse model recapitulated the whole-body SIRT5KO mouse model’s response to the same stress. Previously, we described a potential mechanism of increased mortality due to impaired oxidative metabolism in cardiomyocytes, with a primary lesion at OXPHOS, and an accelerated trajectory of cardiac dysfunction (Figure 14). Surprisingly, we saw no difference in mortality between heart-specific SIRT5KO mice and their littermate controls in response to TAC. One major caveat of this study was the degree of succinylation in the heart-specific SIRT5KO mouse at time of TAC surgery (approximately 2-fold) compared to the degree of succinylation in the whole-body SIRT5KO mouse at time of TAC surgery (approximately 5.5-fold; see Figure 10). Based on these data, we propose two models to describe the effect of SIRT5 on cardiac function.

One model that could explain the different response to TAC in the whole-body and heart-specific SIRT5KO models is that the effect is due to a whole-body or multi-tissue role of SIRT5. Although succinylation increases dramatically in the heart in whole-body SIRT5KO mice, it also increases greatly in the kidney, brain, and brain adipose tissue (Figure 2). Cardiac function is intricately linked to kidney function, and thus,
Increased mortality with TAC in whole-body SIRT5KO mice could be a result of Sirt5 ablation in both the heart and kidney. Indeed, there is evidence that SIRT5 plays a role in regulating ammonia production by desuccinylation (and activation) of glutaminase whereby loss of SIRT5 leads to increased ammonia production (Polletta et al., 2015). Increased ammonia production can contribute to kidney disease (Chen and Abramowitz, 2014) and could play a role in the response of whole-body SIRT5KO mice to TAC. Additionally, the cardiac hypertrophic response is not exclusive to cardiomyocytes, but also includes an inflammatory response with the infiltration of macrophages and increased fibrosis (Samak et al., 2016). A role for SIRT3 in preventing fibrosis by activity in fibroblasts has recently been described (Sundaresan et al., 2015) and several studies suggest that sirtuins may reduce inflammation (Winnik et al., 2015). The specific role of SIRT5 in the inflammatory response is a nascent area of investigation, but recent studies suggests that SIRT5 suppresses the pro-inflammatory response in macrophages (Wang et al., 2017). Therefore, a possible explanation for lack of difference in response to TAC in cardiomyocyte-specific SIRT5KO mice compared to WT controls could be an improved inflammatory response that is impaired in whole-body SIRT5KO mice with TAC.

In another model, we predict that the degree of succinylation may influence the response to TAC. Given the differences in succinylation at the time of TAC surgery in the whole-body SIRT5KO model (Hershberger et al., 2017a) and the heart-specific
SIRT5KO model (Figure 19), it is possible that succinylation of enzymes is not sufficient to alter overall oxidative metabolism in response to TAC. Indeed, when we look at specific targets of SIRT5 in the heart, it appears that succinylation continues to increase between 15-weeks and 31-weeks post SIRT5 depletion (Figure 20). To address this caveat, mice could be aged for more than 30 weeks after Sirt5 ablation before TAC surgery in order to increase protein lysine succinylation. However, this would increase the age of the mouse at time of surgery from 12-20 weeks to close to 40 weeks of age. This itself could pose problems in comparing the heart-specific and whole-body models of the same age as whole-body SIRT5KO mice aged to 39 weeks exhibit cardiac dysfunction compared to controls (Sadhukhan et al., 2016). Another way to address this caveat could be to develop a muscle-specific SIRT5KO by crossing SIRT5fl/fl and MCK-Cre mice. Expression of MCK occurs in skeletal and cardiac muscle, expression begins embryonically, and is constantly high throughout life (Brüning et al., 1998). This model might more accurately reflect cardiac succinylation in the whole-body SIRT5KO model, although the acylation profile over time of studies using the muscle-specific SIRT3KO model have not been reported (Fernandez-Marcos et al., 2012; Martin et al., 2017).

In this study, we present the development and characterization of a novel heart-specific SIRT5KO mouse model that may aide in disentangling the effects of SIRT5 and succinylation. Together, our data suggests two possibilities regarding the role of SIRT5 in cardiac function. First, the response to TAC is dependent on SIRT5 in multiple tissue
or cell types. Second, succinylation is required to reach some saturating levels before the overall effect of impaired oxidative metabolism with TAC is observed. Future studies will determine the response to TAC in a setting of saturated succinylation in a heart-specific SIRT5KO mouse model in order to definitively determine if the whole-body SIRT5KO response to TAC is a cardiomyocyte intrinsic effect of SIRT5.

4.4 Summary

The goal of this part of my dissertation was to determine if increased mortality in response to TAC is due to a cardiac-intrinsic or whole-body effect of SIRT5. To answer this question, I generated and characterized a heart-specific, tamoxifen-inducible SIRT5KO mouse model. In collaboration with the Duke Cardiovascular Physiology Institute, we exposed heart-specific SIRT5KO mice to the same chronic TAC as in Chapter 3 of this dissertation. We found that the whole-body SIRT5KO phenotype did not recapitulate the heart-specific SIRT5KO phenotype. However, I uncovered an interesting observation regarding succinylation in this heart-specific SIRT5KO model; protein succinylation on whole cell heart extracts continued to increase over the course of months, even though SIRT5 was maximally depleted within a few weeks. I sought to investigate how the landscape of protein succinylation changed weeks to months after Sirt5 ablation and found that a subset of succinylation sites continue to increase in succinylation. In Chapter 3, I hypothesized that the overall succinylation landscape
contributed to impaired oxidative metabolism in whole-body SIRT5KO mice with TAC. However, my work in the heart-specific SIRT5KO model may suggest that there are specific targets of SIRT5 that contribute to the phenotype. This is further discussed in Chapter 5 of this dissertation.
5. Conclusions

The purpose of this dissertation was to determine the physiological role of SIRT5 and protein succinylation in the heart. This is an important area of investigation because the sirtuins are recognized as a family of proteins that promote health and longevity, and further understanding their physiological roles could pave the way for potential therapeutic applications. Importantly, the enzymatic activities of SIRT5 are well established, but how SIRT5 contributes to cardiac function and metabolism is not well understood. I hypothesized that a cardiac stress would be required to observe a cardiac phenotype in the whole-body SIRT5KO mouse model. Additionally, I predicted that multiple sites of protein succinylation would contribute to cardiac or metabolic dysfunction. The experiments in this dissertation were designed to address these questions.

In Chapter 3, I exposed whole-body SIRT5KO mice to 16-week TAC and observed increased mortality in SIRT5KO mice compared to WT littermate controls. This lead me to investigate the cause of increased mortality. In collaboration with the Duke Cardiovascular Physiology Core, we used echocardiogram and invasive hemodynamic measurements to characterize cardiac function. Although echocardiogram data showed that SIRT5KO mice develop exaggerated hypertrophy after 4 weeks of TAC, this observation was not supported by other measurements of cardiac hypertrophy included the ratio of left ventricle weight to body weight. Despite the striking phenotype of
increased mortality in SIRT5KO TAC compared to WT TAC mice, we surprisingly found few differences in cardiac function. However, using a combined metabolomics and proteomics strategy, we discovered that SIRT5KO mice appeared to be on a trajectory of accelerated heart failure due to defects in oxidative metabolism. Together, this work suggests that one physiological role of SIRT5 in the heart is to maintain oxidative metabolism. This lead us to ask if this was a cardiac-intrinsic role of SIRT5.

To address this question, I developed a heart-specific, tamoxifen-inducible SIRT5KO mouse model. We exposed these mice to a 16-week TAC and found no difference in survival. However, the whole-body SIRT5KO and heart-specific SIRT5KO mouse models had different profiles of protein succinylation. While the whole-body SIRT5KO heart maintained a high level of succinylation (~5.5-fold higher compared to WT controls), protein succinylation in the heart of the heart-specific SIRT5KO model increased for months after tamoxifen-induced Sirt5 ablation. Succinyl proteomics showed that a subset of succinylation sites had increases in succinylation between 15- and 31-weeks post Sirt5 ablation, including three validated targets of SIRT5 in the heart.

In the final chapter of this dissertation, I synthesize the major findings of my work with the current work in the field, and suggest future directions of my work.
5.1 Succinylation does not appear to regulate metabolism

The major observations regarding protein succinylation in my work include that protein succinylation decreases in whole-body SIRT5KO hearts with TAC (Figure 10), protein succinylation accumulates over the course of months after Sirt5 ablation (Figure 16), and succinylation in the heart is overrepresented in pathways of oxidative metabolism regardless of the model (Figure 10 and Figure 20).

One major question in the field is whether acylation regulates metabolism, or is a consequence of normal metabolism and/or shifts in metabolism. Recent work has proposed that alterations in acylation in the newborn heart contribute to shifts in metabolism including a decrease in glycolysis and an increase in fatty acid oxidation (Fukushima et al., 2016). The authors show that acetylation and succinylation increased in newborn rabbit hearts from post-natal day one to post-natal day 21. The data presented from this study is largely correlative, and changes in transcriptional regulation, such as an increase in PPARα protein expression could also explain the regulation of shifts in metabolism. The authors show that there was an increase in acetyl-CoA generation from fatty acid oxidation over this time period, which could explain the concomitant increase in protein acetylation. While succinyl-CoA is not measured, it is also plausible that shifts in metabolism (from glycolytic to more oxidative in the newborn heart) contribute to increased succinyl-CoA that leads to protein succinylation. Therefore, while this study suggests that acylation leads to shifts in
metabolism, I find that their data could also support a model where shifts in metabolism lead to increases in acyl-CoA pools and increased protein lysine acylation.

The data presented in my dissertation also provides evidence that supports the hypothesis that protein acylation is a result of shifts in metabolism and does not serve to regulate metabolism. In the whole-body SIRT5KO mouse under basal conditions, we observe dramatic hypersuccinylation compared to WT controls, but no change in metabolite profiles in fatty acid oxidation (Figure 11) or glucose oxidation (Figure 12), suggesting that succinylation does not regulate metabolism under these conditions. In contrast, when metabolism shifts under the TAC condition, I observe a concomitant decrease in protein succinylation in SIRT5KO mouse (Figure 10). Shifts in metabolism away from oxidative metabolism to glycolytic metabolism would predict a decreased succinyl-CoA pool and could explain the decrease in succinylation in the absence of a desuccinylase. Although succinyl-CoA is not directly measured, we do see an overall decrease in TCA cycle metabolites (Figure 12).

Secondly, the heart-specific SIRT5KO mouse model demonstrates that protein succinylation occurs slowly. Given that succinyl-CoA forms an extremely reactive intermediate (Wagner et al., 2017), I originally hypothesized that a significant increase in protein succinylation would occur within days after SIRT5 depletion. However, upon further thought, it makes sense that succinyl-CoA available for protein succinylation would not be in excess as metabolism is under tight regulation to maintain homeostasis.
(Ghanta et al., 2013). Therefore, I predict that the slow accumulation of protein succinylation after SIRT5 depletion is due to low availability of succinyl-CoA for the fate of succinylation (instead of further oxidation), and the absence of another desuccinylase. This observation supports the hypothesis that acylation occurs nonenzymatically with increased acyl-CoA availability, and that the sirtuin family of proteins acts to remove these modifications to relieve protein carbon stress (Wagner and Hirschey, 2014).

Interestingly, it appears as though there is some level of protein succinylation saturation as we see no change in succinylation with age in adult mice.

Lastly, I found that, in the heart, protein succinylation occurs on enzymes in oxidative metabolic pathways. This occurred in both the whole-body SIRT5KO (Figure 10) and heart-specific SIRT5KO (Figure 20) mouse models. So far, there is no evidence that sirtuins recognize specific protein motifs as targets of deacylation (Yuan and Marmorstein, 2012), suggesting that lysine sites that are targeted by sirtuins for deacylation may be targeted due to their exposure on the protein surface. It is plausible that sites of protein lysine acylation are identified by chemical properties, such as a depressed pKa values (Ghanta et al., 2013), and that acylation occurs to a greater degree on residues that are chemically poised to accept an acyl modification. Given that there appears to be little preference for specific substrates, it is of interest to ask why the main targets of succinylation occur on pathways of oxidative metabolism. This could be due
to that fact that the main metabolic pathways in the heart are fatty acid oxidation, the TCA cycle, and OXPHOS; and that they are in close proximity to the succinyl-CoA pool.

Based on the synthesis of my data regarding protein succinylation, I suggest that succinylation does not serve as a mechanism to regulate metabolism. However, as discussed in Chapter 1, a number of studies have shown that succinylation on specific lysine residues can decrease the activity of the enzyme. Major caveats to these studies include that enzymatic activities are often tested in ex vivo conditions, and not in the context of whole animal metabolism and physiology. Succinyl proteomics in the whole-body SIRT5KO mouse heart indicated that a number of enzymes described as being regulated by SIRT5 mediated desuccinylation (i.e. SDH and HADHA) are hypersuccinylated in this model, but we observe no changes in whole cell metabolism. This suggests that in the context of organismal metabolism, hypersuccinylation of enzymes is not sufficient to alter metabolism. This could be due to a low stoichiometry of enzyme succinylation and is an area of study that requires further investigation. Previous studies have shown that the stoichiometry of mitochondrial acetyl-lysine modifications is low (Baeza et al., 2014; Weinert et al., 2014). While hypersuccinylation does not affect cardiac function or metabolism under basal conditions, there appears to be an effect of succinylation under TAC. The next section discusses the consequence of aberrant hypersuccinylation in the heart.
5.2 Physiologic role of SIRT5 may not depend on its desuccinylase activity

The work in this dissertation describes several important findings regarding the physiological role of SIRT5 and protein succinylation in cardiac function. First, we confirm that there are no cardiac phenotypes in SIRT5KO mice under basal conditions in either a whole-body (Figure 7, Table 2 and Table 3) or heart-specific SIRT5KO mouse model (Table 4 and Table 5). Chronic pressure overload results in increased mortality (Figure 6) in the whole-body SIRT5KO mouse model, but this phenotype is not repeated in the heart-specific SIRT5KO mouse model under the conditions tested (Figure 18). Here, I suggest that saturating levels of hypersuccinylation could be necessary to reveal the phenotypes observed in the whole-body SIRT5KO mouse. Additionally, I suggest an alternative hypothesis, that the phenotype observed in the whole-body SIRT5KO mouse is due to a role of SIRT5 that is not dependent on its desuccinylation activity.

Major pathways that are succinylated in the absence of SIRT5 include fatty acid oxidation and the TCA cycle, and it appears that a main function of SIRT5 is to maintain oxidative metabolism by its desuccinylation activity (discussed in Chapter 3 of this dissertation). One question that remains in the field is whether the effect of hypersuccinylation is due to specific SIRT5 targets, or the sum off all succinylation events. Succinyl proteomics in the whole-body SIRT5KO heart revealed that pathways in oxidative metabolism contain many proteins with multiple sites of succinylation. However, one caveat of this IPA analysis is that only one site of succinylation per
protein is analyzed. When we examine individual pathways, such as fatty acid oxidation (Figure 11), we find that a majority of identified sites of lysine succinylation have small changes in succinylation when SIRT5 is ablated. In contrast, only a few sites of succinylation increase over 100-fold in SIRT5KO mouse hearts. These sites of succinylation may influence enzyme function. Similarly, comparing the fold change (KO/WT) in succinylation between 15-weeks and 31-weeks post Sirt5 ablation shows that while most sites of succinylation do not change significantly, a number of lysine residues have increases in succinylation between these time-points (Figure 20). It is possible that hypersuccinylation at these sites decrease enzyme activity and therefore SIRT5 desuccinylation serves to maintain enzymatic activity. This hypothesis is supported by other work on the physiological role of SIRT5 in the heart. Of the 28 sites of succinylation on HADHA, one group found that the mutation of one lysine to arginine (K351) led to a significant loss of HADHA function (Sadhukhan et al., 2016). This site of succinylation was only identified in the SIRT5KO mouse heart, but the KO/WT fold change is high in my whole-body SIRT5KO proteomics data, and one of the sites that continues to increase between 15-weeks and 31-weeks post SIRT5 depletion in the heart-specific SIRT5KO proteomics data (Figure 20). Together, these data suggest that the absence of a phenotype in heart-specific SIRT5KO mice could be due to lack of succinylation at specific sites sufficient to inhibit enzyme activity.
Interestingly, even in the whole-body SIRT5KO mouse model where succinylation is saturated, we observe no cardiac phenotypes until a cardiac stress that causes shifts in metabolism is induced. This suggests that any effect of succinylation under basal conditions is insufficient to be detrimental to metabolic flux. When we expose whole-body SIRT5KO animals and their littermate controls to TAC, we see no difference in signaling (Figure 8) or transcriptional regulation of metabolic pathways (Figure 11 and Figure 12), suggesting that hypersuccinylation in SIRT5KO mice is what causes an accelerated progression of metabolic dysfunction. However, it is unclear how the cardiac stress of pressure overload affects SIRT5 function, and it is possible that a desuccinylation-independent role of SIRT5 may be responsible for the increased mortality in SIRT5KO mice exposed to TAC.

We see no evidence that SIRT5 protein expression or its desuccinylase activity changes with TAC in WT mice. In fact, *Sirt5* mRNA decreases in WT mouse hearts 4 weeks after TAC. One possible way that sirtuins are regulated is through sensing NAD⁺ levels (discussed in Chapter 1). However, in the WT mouse heart after 4 weeks of TAC, the NAD⁺/NADH ratio is unchanged (Figure 14), suggesting that SIRT5 is regulated in a different way in the TAC model. The transcriptional regulation of mitochondrial sirtuins is an understudied area of investigation. SIRT5 has been shown to be induced by PGC1α overexpression, and this response is attenuated when by ERRα knockdown (Buler et al., 2014). In support of this, I have cloned the Sirt5 promoter and shown that it is activated
by ERRα (see Appendix B). Interestingly, ERRα is downregulated after TAC and recent studies have shown that ERRα<sup>−/−</sup> mice develop significant cardiac dysfunction due to energetic deficiencies in ATP and PCr compared to control mice after 7 days of TAC (Huss et al., 2007). ERRα is an important regulator of fatty acid oxidation, as one of its targets is Acadm, which encodes the protein for the initial step in fatty acid oxidation (Audet-walsh and Giguère, 2015). The downregulation of ERRα after TAC is consistent with decreased fatty acid oxidation. Given that Sirt5 is under the regulation of ERRα, and that ERRα is decreased in TAC, it seems possible that this regulatory mechanism is responsible for decreased Sirt5 mRNA with TAC. However, even with downregulation of Sirt5, we observe no changes in succinylation or evidence of decreased SIRT5 activity in WT animals with TAC. Only in the complete absence of SIRT5 do we observe increased mortality and decrements in oxidative metabolism with TAC. Together, these observations suggest that decreased Sirt5 mRNA expression with TAC is due to canonical transcriptional changes that occur in pressure-overload induced-hypertrophy, and that SIRT5 desuccinylase activity is not changed under the condition of TAC.

One alternative hypothesis that could explain our data is that increased mortality is not due to desuccinylation activity of SIRT5, but could be due to an undiscovered effect of SIRT5 in multiple tissue types. The cardiac hypertrophic response is not exclusive to cardiomyocytes, but also includes an inflammatory response with the infiltration of macrophages and increased fibrosis (Samak et al., 2016). A role for SIRT3
in preventing fibrosis by activity in fibroblasts has recently been described (Sundaresan et al., 2015) and several studies suggest that sirtuins may reduce inflammation (Winnik et al., 2015). The specific role of SIRT5 in the inflammatory response is a nascent area of investigation, but recent studies suggest that SIRT5 suppresses the pro-inflammatory response in macrophages (Wang et al., 2017). Therefore, a possible explanation for lack of difference in response to TAC in cardiomyocyte-specific SIRT5KO mice compared to WT controls could be an improved inflammatory response that is impaired in whole-body SIRT5KO mice with TAC, but preserved in the heart-specific SIRT5KO mouse exposed to TAC. The role of SIRT5 in different tissue types in an important area of future investigation.

5.3 Future directions

There remain some outstanding questions about the role of SIRT5 and protein succinylation in cardiac function and metabolism that guide the future directions of this dissertation work. Previous work in the field and the studies presented here suggest that cardiac function and metabolism may be impacted only in conditions when Sirt5 ablation from the germline causes aberrant hypersuccinylation that impacts metabolic flexibility in models of cardiac stress. In order to understand the translational implications of this work, it will be important to determine if human cardiac disease models also show protein hypersuccinylation. Protein acetylation has been shown to be
present in cardiac diseases such as heart failure and have direct effects on cardiac metabolism (Horton et al., 2016). If protein succinylation also increases in human cardiac disease models, or SIRT5 activity appears to decrease it would be of interest to further explore the therapeutic potential of SIRT5 activation through strategies such as boosting NAD+$^+$ levels. However, in the absence of changes in SIRT5 activity or protein succinylation, it would suggest that the contribution of SIRT5 and protein succinylation to cardiac pathology is minimal, and perhaps not a therapeutic target to further investigate.

In addition, there remain some further experiments to perform in the genetic mouse models described in this work to fully understand the role of SIRT5 and succinylation in response to stress. In the WT TAC mouse model, we see no evidence of changes in succinylation. However, this does not exclude the possibility that SIRT5 desuccinylates specific sites that are essential to preserving normal enzyme function. Performing succinyl proteomics in the WT and SIRT5KO mice hearts under sham and TAC conditions will allow us to answer this question. Using the succinyl proteomics generated in this dissertation, we can predict sites of succinylation that may decrease enzyme activity and determine if these sites are specifically targeted by SIRT5 under chronic pressure overload.

Finally, performing TAC surgery in a heart-specific SIRT5KO model where the succinylation landscape is equivalent to the succinylation landscape in the whole-body
SIRT5KO mouse model will allow for a definitive answer as to whether the phenotypes observed in the whole-body SIRT5KO model are due to a whole-body effect, a cardiac-intrinsic direct effect of SIRT5, or a cardiac-intrinsic indirect effect of hypersuccinylation. Further characterizing metabolic shifts in the heart-specific SIRT5KO model under TAC will determine if impaired oxidative metabolism is a phenotype that is recapitulated in this mouse model.
Appendix A: Effect of Nnt in a Sirt5−/− background

Cardiac function data presented in Chapter 3 was performed on C57BL/6 mice with a mixed nicotinamide nucleotide transhydrogenase (Nnt) background. Nnt encodes an enzyme of the inner mitochondrial membrane that couples NADPH regeneration with proton translocation across the mitochondrial membrane. The activity of this enzyme is important in maintaining redox homeostasis in the mitochondria. Studies have revealed the presence of mutations in Nnt including a missense mutation in exon 1 and a multi-exon deletion in exons 7-11 that results in a truncated and non-functional NNT protein (Toye et al., 2005). As a result of this Nnt mutation, C57BL/6J mice have metabolic redox abnormalities including higher rates of H₂O₂ release, a higher susceptibility to undergo Ca²⁺-induced mitochondrial permeability transition, and an inability to sustain reduced NADP and glutathione (Ronchi et al., 2013). A recent study comparing C57BL/6N (NntWT) and C57BL/6J (NntMut) cardiac response to transverse aortic constriction revealed a role for NNT in causing oxidative stress in heart failure due to a reversal of the enzyme activity (Nickel et al., 2015). Compared to the C57BL/6J mice, the C57BL/6N mice had decreased left ventricle ejection fraction, more cardiac fibrosis, increased lung fluid, and more ROS formation after 6 weeks of TAC as well as decreased survival (Nickel et al., 2015).

Given the effect of NNT on cardiac function with TAC, we sought to determine if the effect of SIRT5 with TAC was different between different NNT backgrounds. We
went back and genotyped all experimental animals for *Nnt* and found that we had a mix of *Nnt* genotypes present with the majority of animals being heterozygous for the *Nnt* mutant form. First, we separated mice based on their *Nnt* genotype and generated Kaplan-Meier survival curves of WT and SIRT5KO mice over the course of a chronic 16-week TAC study (Figure 21). We found that the trend of reduced mortality in SIRT5KO mice compared to WT mice with a mixed *Nnt* background (Figure 21 A) was consistent when we separated by *Nnt* genotype (Figure 21 B-D). Interestingly, we did not see a difference in survival in WT *Nnt<sup>WT/WT</sup>* and WT *Nnt<sup>Mut/Mut</sup>* mice (Figure 21 B and D) whereas Nickel et al. reported a decrease in survival in C57BL/6N compared to C57BL/6J mice. However, we only have a small group of mice in these groups, and the single WT *Nnt<sup>WT/WT</sup>* mouse was removed from the study due to severe fight wounds approximately 8 weeks post-TAC (Figure 21 D). The median survival of SIRT5KO TAC mice was less than the median survival of WT TAC mice regardless of *Nnt* genotype (Figure 21 E). Given these findings, we decided that grouping the WT mice and SIRT5KO mice together independent of *Nnt* genotype was sound given that we did not see any differences in survival trends when separating by *Nnt* genotype.
Figure 21: Effect of \textit{Nnt} on survival post-TAC

(A) 16-week survival curve of all animals in study (see also Figure 6). (B) 16-week survival of mice with mutant \textit{Nnt}. (C) 16-week survival of mice with heterozygous for mutant \textit{Nnt}. (D) 16-week survival of mice with wild type \textit{Nnt}. (E) Table comparing the median survival of WT and SIRT5KO animals with different \textit{Nnt} backgrounds during chronic TAC.

<table>
<thead>
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<th>\textit{Nnt} Background</th>
<th>WT</th>
<th>SIRT5KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mut/Mut)</td>
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</tr>
<tr>
<td>(WT/Mut)</td>
<td>10.3</td>
<td>2.4</td>
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<tr>
<td>Combined</td>
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<td>2.4</td>
</tr>
</tbody>
</table>
Next, we looked at the effect of NNT on wall thickness, fractional shortening, and ejection fraction 4 weeks post-TAC in WT and SIRT5KO mice (Figure 19). Consistent with Nickel et al., we observe no difference in wall thickness 4 weeks post-TAC in WT mice when comparing Nnt genotypes (Figure 22 A). Additionally, we observe no different in wall thickness 4 weeks post-TAC in different Nnt backgrounds in SIRT5KO mice (Figure 22 B). These data suggest that cardiac morphological response to TAC is independent of Nnt genotype. Additionally, these observations confirm that the increased wall thickness in SIRT5KO mice compared to WT mice 4 weeks post-TAC is an effect of SIRT5 (see Figure 7). Nickel et al. report a decrease in ejection fraction in C57BL/6N mice 6 weeks post-TAC, but no change in ejection fraction in C57BL/6J mice 6 weeks post-TAC. When we combine all mice independent of Nnt genotype, we observe a trend of decreased fractional shortening in WT and SIRT5KO mice 4 weeks post-TAC with no difference in Sirt5 genotype. Using pressure volume loop hemodynamic analysis, we observe a strong trend of decreased ejection fraction in the SIRT5KO mice, but preserved ejection fraction in WT mice. In order to determine if the differences we observed were due to different Nnt genotypes, we again separated the WT and SIRT5KO TAC groups into three groups representing the three possible Nnt genotypes present in our C57BL/6 mixed background. Interestingly, in the Sirt5+/+ condition, the WT Nnt^{WT/Mut} group has decreased fractional shortening compared the Nnt^{Mut/Mut} and Nnt^{WT/WT} group, but there is no difference in fractional shortening between the Nnt^{Mut/Mut} and Nnt^{WT/WT}
groups (Figure 22 C). Although we present the statistical analysis here, it is important to note that this comparison is slightly underpowered (with $\alpha=0.05$, power=0.8, observed differences require $n=4$ per group). Based on observations from Nickel et al., we would expect the heterozygous $Nnt$ group to behave intermediately between the mutant $Nnt$ and wildtype $Nnt$ genotypes (Nickel et al., 2015). Therefore, we conclude that adding more animals to the SIRT5KO $Nnt^{WT/WT}$ group will be necessary to determine if this relationship is real. When we use hemodynamics to more accurately determine ejection fraction, we find no differences between $Nnt$ genotype, though there are fewer mice represented in these groups, and none with the $Nnt^{WT/Mut}$ genotype, causing greater variability in the groups (Figure 22 E). Finally, we see no difference in fractional shortening (Figure 22 D) or ejection fraction (Figure 22 F) in SIRT5KO TAC mice when separated by $Nnt$ genotype.

Overall, our functional cardiac characterization data is variable. We attribute this to the inherent variability of the TAC surgery. Based on the data presented here, another cause of variability could be $Nnt$ genotype. However, it will take a greater number of animals to fully sort out the effects of $Nnt$ versus SIRT5. That being said, we do not see any genotypic differences in cardiac function between the WT TAC and SIRT5KO TAC mice and therefore combined $Nnt$ genotypes, which may increase our probability of a type I error (i.e. a false negative). Moving forward, it was decided to generate mice for future mechanistic studies to be on the same $Nnt$ background. For the mechanistic
studies presented in chapter 3, all animals were on the C56BL/6J background in order to be on the same background as the heart specific SIRT5KO model discussed in chapter 4.

![Graphs](Image)

Figure 22: Effect of *Nnt* on cardiac morphology and function in WT and SIRT5KO mice 4 weeks post-TAC

Wall thickness (WTh) in (A) WT and (B) SIRT5KO mice 4 weeks post-TAC separated by *Nnt* genotype. Percent fractional shortening (FS) in (C) WT and (D) SIRT5KO mice 4 weeks post-TAC. Data was collected using echocardiogram (A-D). Ejection fraction in (E) WT and (F) SIRT5KO mice 4 weeks post-TAC. Data was collected using pressure volume loop hemodynamic analysis (E and F). One-way ANOVA with multiple corrections in all panels except E where a Student’s t-test was performed because only two groups were represented. *p≤0.05.
Appendix B: Transcriptional Regulation of SIRT5

Because dysregulation of sirtuins contributes to several diseases, it is important to understand how the sirtuins are regulated. As described in the introduction of this dissertation, one way that sirtuins are regulated is through the availability of NAD+.

Another way that sirtuins are regulated is transcriptionally, and a number of transcription factors have been identified that regulate SIRT1 positively and negatively (Buler et al., 2016). Little is known about how SIRT5 is transcriptionally regulated. SIRT5 has been shown to be induced by PGC-1α overexpression, and this response is attenuated when by ERRα knockdown (Buler et al., 2014). However, it has not been shown that these nuclear factors are direct transactivators of SIRT5. Here, we describe preliminary studies characterizing and cloning the putative promoter of SIRT5 and suggest that SIRT5 is directly activated by ERRα.

To determine the most likely region of the Sirt5, promoter, an analysis using a variety of online resources was performed. We looked for regions of homology to the mouse genome, regions where H3K27 acetylation was likely (as this mark is often found near active regulatory elements), and regions of CpG islands, which are also indicative of regions of active regulatory elements. The UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) provides reference sequence information for a variety of genes. It is also useful for determining the active region of the sirtuin promoters as it graphically shows regions of H3K27Ac. For SIRT5, this area is about 1 kb
downstream of the transcription start site (TSS) and 1kb upstream of the TSS (Figure 23 A). The region 1 kb downstream of the TSS appears to be highly conserved in Rhesus, and well-conserved in mouse, dog, and elephant, suggesting that this region may be a promoter region of SIRT5 (Figure 23 B). Lastly, we analyzed putative CpG islands using Cpgplot (EMBOSS). Sequences rich in the CpG pattern are resistant to methylation and tend to be associated with genes that are frequently switched on, indicative of active promoter regions. CpG islands lie 400 base pairs upstream and downstream of the SIRT5 TSS (5000 bp represents TSS; Figure 23 C). Based on this data, it appears that a promoter region of SIRT5 is within one thousand base pairs from the TSS.

We decided to clone a region of SIRT5 that encompassed about two thousand base pairs — one thousand base pairs downstream of the TSS and 800 base pairs upstream of the TSS. We used a forward primer with the sequence 5’-GGTAACACCTGCAGGAATGAGAAG-3’ and a reverse primer with the sequence 5’-CATCACTCCCAGGTGTCTCCAC-3’. Human genomic DNA (Promega) was used as a template and MyFi DNA polymerase (Bioline) were used in the PCR reaction to amplify the putative SIRT5 promoter region. The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen) and cloned into a pCR2.1-TOPO TA vector (TOPO TA Cloning Kit, ThermoFisher Scientific). The vector containing the SIRT5 promoter was transformed into GC5 Competent cells (Sigma Aldrich) according to manufacturer instructions and plated overnight on LB/Amp plates. Colonies were picked, grown in 3
mL LB/Amp cultures, and purified using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer instructions. Digest with EcoRI-HF (New England Biolabs) was used to verify correct insert size. Approximately 2 µg of pCR2.1 TOPO plasmid with verified SIRT5 insert was digested with XhoI (New England Biolabs) and HindIII (New England Biolabs). 100 ng of digested SIRT5 was ligated into 100 ng of digested pGL4.21 (Promega) vector for luciferase assays. Dual-Luciferase Reporter Assay System (Promega) was used according to manufacturer instructions. We found that the SIRT5 promoter is activated by the transcription factor ERRα (Figure 23 D). This transcription factor is active in tissues with high use of fatty acids such as heart, kidney, and skeletal muscle.
Figure 23: Identification and verification of putative Sirt5 promoter

UCSC genome browser screen shots of (A) H3K27 Acetylation and (B) conservation mapped to SIRT5. (C) Screen shot of putative CpG islands where 5000 bp represents the TSS. (D) Luciferase assay showing that the cloned putative SIRT5 promoter is activated by the transcription factor ERRα.
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