12-2017

INSIGHTS INTO THE THERAPEUTIC POTENTIAL OF SALT INDUCIBLE KINASE 1: A NOVEL MECHANISM OF METABOLIC CONTROL

Randi Fitzgibbon

INSIGHTS INTO THE THERAPEUTIC POTENTIAL OF SALT INDUCIBLE KINASE 1:

A NOVEL MECHANISM OF METABOLIC CONTROL

by

Randi Nicole Fitzgibbon

APPROVED:

_____
Rebecca Berdeaux, Ph.D.
Advisory Professor

_____
Vihang Narkar, Ph.D.

_____
Guangwei Du, Ph.D.

_____
Edgar Walters, Ph.D.

_____
Pramod Dash, Ph.D.

APPROVED:

_____
Dean, The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

INSIGHTS INTO THE THERAPEUTIC POTENTIAL OF SALT INDUCIBLE KINASE 1: A NOVEL MECHANISM OF METABOLIC CONTROL

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Randi Nicole Fitzgibbon
Houston, Texas

December 2017

Dedication

This dissertation is dedicated to my family and mentors:

Ryan and Liam Fitzgibbon, Judy Stewart and Wayne Shuffield, Kathy Rose,

and Dr. Rebecca Berdeaux.

Each of these individuals has given me unique gifts and has inspired me to always strive for solid, honest work that reflects nothing less than my best efforts.

Acknowledgements

“Appreciation is a wonderful thing.
It makes what is excellent in others belong to us as well”
-Voltaire

Several acknowledgments are in order to properly credit my mentors, family, and friends who have supported me through this long journey either through direct scientific input or through more intangible means. First and foremost, I want to express immense gratitude to my mentor, my hero, Dr. Rebecca Berdeaux. It wasn’t until working in Rebecca’s lab as a research technician that I discovered my passion for science. Rebecca did not just teach me how to maneuver a pipet with precision; she taught me intangible lessons that I will always hold dear. Three major lessons come to mind: 1. Things aren’t always what they seem on the surface and the most obvious answer is not always (or even typically) the correct answer. 2. All the natural talent in the world can never beat resilience, rigor, and persistence (I’ve recently concluded that “Doctor of philosophy” could also be known as “Doctor of persistence”). 3. In both science and life, you can choose to focus on the battles or the victories- sometimes the battles are long and frequent, but they never equal our sweetest victories. I hold on tight to my victories and accomplishments in part because Rebecca taught me the importance of gratitude and quiet strength as weapons during the toughest battles. There is no doubt I would not be in this position without the mentorship and friendship of Dr. Rebecca Berdeaux.

Several other mentors have played pivotal roles in my journey towards this degree. To my advisory committee who was there all along the way, Dr. Vihang Narkar, Dr. Guangwei Du, Dr. Edgar Walters, and Dr. Pramod Dash: I greatly benefitted from

your thoughtful comments and suggestions (even when it meant I had a lot more work to accomplish). You never sat idly in meetings to simply check off a box. The level of engagement and enthusiasm you each shared with me has most certainly improved the quality of work presented in this dissertation and has undoubtedly made me a better scientist. I am completely grateful for this. Dr. George Rodney also played an important role in my thesis work and in my development as a scientist. He taught me a different, more refined, perspective of muscle physiology than I had held before. He also taught me how to perform some of the coolest experiments in my repertoire. Dr. Neal Waxham fortuitously became a vital mentor for me as I worked to address the final aims of my dissertation work. I have never met a more charitable an enjoyable collaborator. I enjoyed our scientific conversations more than you know and am in debt to you for sharing even just a tiny piece of your unmatched prowess in high tech microscopy.

A great deal of the work herein was accomplished as a team effort. Dr. Nicholas Justice played a significant part in the development of the SIK1 conditional knockout mouse line that has proven to be an important model and now significant contribution to our field of study. Additionally, Nick has provided invaluable scientific feedback and critique through the years and has encouraged me to consider the strengths and weaknesses of my work from a unique perspective. His ideas have certainly enhanced the power of this dissertation research. Courtney Leiter and Christopher Robb were laboratory technicians with me in Dr. Berdeaux's lab just prior to my matriculation to GSBS. Both of these individuals contributed to work presented in chapter 3 and were diligent and pleasurable to work beside, even when the blot rockers were still working full force on Friday nights. I admire both Courtney and Chris's technical skills and hope

to somehow emulate the same sort of team dynamic in future professional settings. Several other lab members have contributed greatly to the research presented herein: Dr. Dmitry Akhmedov was a co-first author on the work described in chapter 3 of this dissertation and also contributed to work described in chapters 4 and 5. His work is represented in nearly half the published figures in our 2013 PNAS publication. He played a pivotal role in the SIK1 mutant experiments described in chapter 3 and was the lead researcher in demonstrating PKA-dependent phosphorylation of SIK1 at a novel sequence site. Dr. Mark Nixon was a co-first author with me in the work published in Molecular Metabolism in 2016 (presented in chapter 5). Mark completed most of the metabolic testing of the global SIK1 knockout mice and contributed greatly to the scientific discussion and ideas presented in this work. In this same study, Dr. Jingqi Fu performed the experiments presented for SIK1 liver-specific knock out mice and the primary hepatocyte experiments. Kavitha Rajendran and Maria Mendoza-Rodriguez contributed considerably to this study (and in Maria's case, the work in chapter 5) by performing experiments and serving as blind, unbiased researchers for skeletal muscle measurements.

It is not lost on me how fortunate I have been to study and research in the Texas Medical Center at The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. This community and institution provides researchers cutting edge technology and is an ideal forum for discussing science and developing collaborations essential for top notch research. I have never felt limited in the experiments I could perform to complete my project and have found world-renowned experts in every corner

who have been willing to discuss my work. I would especially like to acknowledge the Center for Clinical and Translational Sciences (CCTS) who not only provided funding for my research through the TL1 program, but also served as a conduit for establishing a relationship between my laboratory and my clinical mentor Dr. Absalon Gutierrez (UT Physicians- Endocrinology, Diabetes, and Metabolism). The shadowing opportunities I seized in Dr. Gutierrez's office really enlightened my work and underlined the unmet clinical needs in metabolic syndrome and type 2 diabetes fields.

The final mentors I would like to acknowledge are individuals who served unexpected roles in my training. I spent some of my most formative years working for Kathy Anne Rose and received more than just a paycheck. Kathy served (and still serves) as my role model for how an educated and classy woman conducts herself in business and in personal environments. My mother, Judy Stewart, has mentored me from the very beginning. It's no coincidence that I am currently reflecting on the importance of persistence in science and her lifelong philosophy has been "you don't quit something you've committed to doing". Not every day in scientific research has been pleasurable; I spent many nights with aching feet from standing at the bench all day and with a pounding head from frustrating experimental failures; however, quitting was never an option because I knew that just like you can't quit track and field, you can't quit now. My stepfather, Wayne Shuffield, has been an inspiration to me by demonstrating what it takes to excel and lead in a profession. He accepts nothing but the best from himself and has encouraged me to do the same. To

my friends who have served as role models, counsellors, and confidants, I am eternally grateful for your friendship: Kelsey Maxwell, Tanya Baldwin, Lizzy Smith, Alex Holeman, my Crockett girls (Jenn, Heather, and Tamara), Gabriella Burke, and Daisy Thompson-Lake (who specifically encouraged strict dedication through long study/writing sessions at Café Brasil- our home away from home).

Last but certainly not least, I would love to acknowledge my husband, Ryan Fitzgibbon, for supporting me through the years. We have had to sacrifice at times so I could get the last minute results I needed for grants or presentations. You have had plenty of nights eating dinner alone because I was working late and more recently you have taken on an extra load of childcare so Liam's mommy could pursue her dreams. I love and adore you and am unbelievably grateful you continue to stand in my corner.

INSIGHTS INTO THE THERAPEUTIC POTENTIAL OF SALT INDUCIBLE KINASE 1: A NOVEL MECHANISM OF METABOLIC CONTROL

Abstract

Randi Nicole Fitzgibbon

Advisory professor: Rebecca Berdeaux, Ph.D.

Salt inducible kinase 1 (SIK1) has been considered a stress-inducible kinase since it was first cloned in 1999. Continued efforts since this time have been dedicated to characterizing the structure and function of SIK1. Such research has laid the ground work for our understanding of SIK1 action and regulation in tissue and stimuli dependent manners. The fundamental findings of this dissertation continue in this tradition and include investigations of SIK1 regulatory mechanisms in skeletal muscle cells, the cellular and physiological effects of SIK1 loss of function *in vitro* and *in vivo*, and intracellular metabolic and mitochondrial regulation by this kinase. Herein, evidence is provided demonstrating that skeletal muscle SIK1 regulates insulin sensitivity and blood glucose concentrations through mechanism(s) independent of the canonical insulin pathway. Our research addresses many previously unanswered questions about SIK1 action in metabolism and positions SIK1 as a potential therapeutic target for the treatment of metabolic disorders, such as type 2 diabetes, while provoking new questions for future research.

Table of contents

List of Illustrations

List of Tables

Abbreviations

AA: amino acid

ActD: Actinomycin D

ACTH: Adrenocorticotropin hormone

AMPK: AMP- activated protein kinase

CREB: cAMP response element binding protein

CRTC: CREB-regulated transcription coactivator (formerly TORC)

CTX: cardiotoxin

DAG: diacylglycerol

DIO: diet induced obesity

FSK: forskolin

GLUT: Glucose transpoter

HDAC: Histone deacetylase

HFD: high fat diet

IBMX: 3-isobutyl-1-methylxanthine

IGF: Insulin-like growth factor

IRS: Insulin receptor substrate

LKB1: Liver kinase B1 or Serine/Threonine Kinase 11 (STK11)

MEF: Myocyte enhancer factor

OCT: Optimal Cutting Temperature Compound

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

SIK1: Salt inducible kinase 1

TEM: Transmission electron microscopy

T2DM: type 2 diabetes mellitus

Chapter 1: Introduction

1.1 Salt Inducible Kinase 1

1.1.1 Discovery of SIK1

In the pursuit of identifying factors linking adrenal cortex stimulation and steroidogenesis, Wang et. al., led by Dr. Mitsuhiro Okamoto, reported the discovery of a previously un-identified polypeptide of 776 amino acids in 1999 (1). The cDNA was cloned from adrenocortical glands of rats stressed with high salt diets (to promote ACTH stimulation of adrenal cells) but was not present in glands of un-stressed control rats. Further investigation of the protein structure revealed that the protein contained a putative kinase domain and possessed striking similarities to the AMPK family of serine/threonine kinases. For these reasons, the authors termed the newly identified protein “Salt inducible kinase”. In this original publication, the authors demonstrated that ACTH treatment directly stimulates SIK expression in adrenal cells and that SIK indeed possessed enzymatic activity. Additionally, the authors completed histological studies to demonstrate patterns of SIK expression. In the before described conditions, *Sik* mRNA was primarily detected in adrenal glands followed by very low expression in heart, kidney, stomach, lung, testes, and ovary. Researchers later discovered that this pattern of expression provided a narrow perspective on the tissue distribution of SIKs due to the specific model used (see section 1.1.3 for insight into SIK expression in a wide variety of tissues). The identification of a new AMPK-related kinase opened a new field of study that would expand our knowledge of cell signaling pathways and regulation in several tissue types and under a wide variety of stimuli.

1.1.2 Timeline of important SIK1 discoveries

Drawing from the previously established connection between ACTH stimulation and increased cAMP-dependent signaling and gene transcription, researchers soon discovered the newly identified kinase lies within the cAMP pathway as a target of PKA-dependent phosphorylation (2, 3). Phosphorylation of SIK on Ser577 by PKA induces nuclear export of SIK and reduces the ability of SIK to inhibit CREB-dependent transcription (3, 4) of an increasingly large list of genes described over time. Although SIK was established as a negative regulator of CREB-dependent transcription, the molecular mechanism by which this occurs was unclear for several years. In the meantime, significant progress was made in the effort to characterize the domains and putative sites of post-translational modification in SIK and in 2003 additional isoforms of SIK (SIK2 and SIK3) were described (5). The primary topic of this thesis is the characterization of SIK1 but it is important to note that the other SIK isoforms are of significant physiological consequence, both congruent and divergent to the actions of SIK1 depending on tissue and stimulus type.

By 2003, the physiological conditions of SIK1 relevance had begun to diversify. In addition to an adrenal gland transcriptional regulator, SIK1 had been described as a stress responsive protein in brain injury models (6, 7) and a protein over-expressed in liver, fat, and skeletal muscle during obesity (5) (see section 1.2.1 for further discussion on stimuli that promote SIK1 activity). In 2004, research led by Dr. Marc Montminy played a pivotal role in our understanding of how CREB-dependent transcription is turned off by SIKs. This work established a link between SIKs and a CREB transcriptional co-activator, CRTC (formerly TORC) (8). They showed that SIK2 could

phosphorylate CRTC2 at serine 171 in pancreatic β cells which prompted 14-3-3-mediated translocation of CRTC2 out of the nucleus, effectively shutting down CREB-dependent transcription of target genes. This negative feedback mechanism of transcriptional regulation has since been described for all SIK and CRTC isoforms and has been demonstrated to occur in a wide variety of tissues. Indeed, CRTCs remain the best characterized SIK substrates today.

The mechanisms regulating SIK1 action were further elucidated in 2006 with the revelation that the master AMPK-family kinase regulator, LKB1, critically phosphorylates and activates SIK1 (9). Inhibition of LKB1 completely ablates SIK1 phosphorylation of CRTC and leads to uncontrolled transcription of CREB target genes. In 2007, two publications established class II HDACs (HDAC4 and HDAC5) as new SIK substrates and importantly linked SIK regulation of gene transcription to a new transcription factor, MEF2 (10, 11). Much like the case for CRTC, SIK1 can phosphorylate class II HDACs at conserved 14-3-3 sites that promote nuclear exclusion of the transcriptional regulator. Unlike CRTCs, however, class II HDACs are transcriptional repressors of MEF2, so SIK1 action on these proteins promotes gene transcription rather than inhibit, as in the case of CRTC/CREB.

In late 2007, SIKs were then shown to suppress gluconeogenesis in liver cells (12), and SIK1 was described as a promoter of active sodium transport in renal epithelial cells (13). Further diversification of SIK1 tissue distribution and action arose from 2009/2010 publications linking SIK1 action to circadian rhythm regulation in the brain (14), cancer metastasis (15), hypertension (16), and cardiomyogenesis (17). These and other studies demonstrate that SIK1 is a multi-functional protein that is

nearly ubiquitously expressed under the right conditions- an insight that was not clear at the time of SIK discovery in 1999. Additionally, similar SIK1 actions have been described in *c. Elegans* (10) and *Drosophila melanogaster* (18, 19) as those attributed to mammals. These findings demonstrate that SIK is a highly conserved protein.

The first mouse model of SIK1 loss of function was reported in 2012 and identified SIK1 as a critical regulator of E-cadherin expression in mouse lung cells (20). Around this time, our group published work defining more post-translational regulatory mechanisms for SIK1 action (described in chapter 3) and demonstrated a role for SIK1 in muscle cell differentiation (myogenesis) (21). The data we presented in this work prompted us to expand the research tools available for studying SIK1 loss of function because we desired to study SIK1 action in a tissue specific manner. Therefore, we produced the first conditional *Sik1* knockout mouse (published in 2015 and described in chapter 4).

One of the most pivotal publications on SIK1 in recent years came in 2015 when a large screen for potential SIK1 substrates was published (22). In addition to identifying PDE4D (a cAMP inhibitor) as a new SIK1 substrate, this group performed kinase assays on other potential SIK1 targets (based on their reported SIK1 phosphorylation consensus sequence). They provided a list of proteins that can be phosphorylated by SIK1 (refer to section 1.2.2 and Table 1 for more details) which is of potentially monumental benefit to researchers in future work.

In late 2015, we published research that is largely the basis of this thesis. In this work, we presented the first conditional SIK1 knockout mouse line and described our

use of these mice to reveal functions of SIK1 as metabolic regulator (23). This work established the likely clinical effectiveness of inhibiting SIK1 for the treatment of type 2 diabetes and metabolic disorders. Indeed, multiple groups are currently working to develop SIK inhibitors (24-27).

The work completed over the last 18 years characterizing SIK1 has established a model in which SIK1 expression is relatively low in un-challenged states but increases in response to stress to re-establish homeostasis. Some of the most pivotal work on SIK1 has been the defining of regulatory pathways controlling this kinase and the identification of SIK1 effector proteins, both of which are discussed in the following sections.

1.2 Endogenous regulatory pathways of SIK1

1.2.1 Stimuli of SIK1 expression and action

As previously mentioned, SIK1 was originally identified as a kinase up-regulated in response to diets enriched in salt and in response to ACTH cellular stimulation. Since the initial 1999 discovery, several SIK1 inducing stressors have been identified (Table 1). Importantly, the tissue distribution of SIK1 expression is stimulus-dependent, and not all stimuli enhance SIK1 expression or activity in the same tissue pattern. The diversity of SIK1 action among divergent organ systems and pathologies has united scientists from diverse fields of study over a common and sometimes unanticipated interest. It is important we continually update the catalogue of SIK1 stimuli since it is often the case that cells need stress inducers to promote SIK1 expression. A readily available synopsis

of such stimuli, as in Table 1, may prove useful for researchers intending to study this protein.

Table 1. Stimuli of SIK1 expression and activity

Stimulus	Tissue	Effect on SIK1	Citation
High salt diet	Adrenal gland	Increased expression	(1)
ACTH	Adrenal gland	Increased expression	(1)
Membrane depolarization	Brain- hippocampus, cortex	Increased expression	(6)
Forskolin	Ubiquitous	Increased expression	(2)
Fluid percussion	Hippocampus	Increased expression in adults but not pups	(7)
Cellular differentiation	Adipocytes, cardiomyocytes, skeletal muscle cells	Increased expression	(5, 17, 21)
Dexamethasone	3T3-L1 cells	Increased expression	(5)
Db/db mutant mice	3T3-L1 cells	Increased expression	(5)
LKB1 manipulation	Ubiquitous- first shown in HeLa	Loss of LKB1 inhibits SIK1 kinase activity	(9)
Phenylephrine	C2C12 cells	Increased expression	(11)
High intracellular sodium content	Renal epithelial cells	SIK1 association with NK complex and increased NK activity	(13)
TGF-β	Human breast carcinoma cells, keratinocytes	Increased expression; SIK1 found in ALK5/Smad complex	(28)
AICAR	C2C12 cells	Increased SIK1 phosphorylation at Thr182	(29)
CRTC1 nuclear localization	Cortical neurons	Increased expression	(30)
Norepinephrine	Rat pineal gland	Increased expression	(14)
Nocturnal hours-awake state in mice	Rat pineal gland	Increased expression	(14)
Hypertension	Renal proximal tube cells	Increased SIK1 phosphorylation at Thr182	(16)

Muscle injury	Skeletal muscle	Increased expression	(31)
Isoproterenol	Lung epithelial cells; cardiomyocytes	Increased SIK1 phosphorylation at Thr182; increased expression (un-published)	(32) and un-published
Cocaine	Rat striatum	Increased SIK1 phosphorylation at Thr182; increased SIK1 phosphorylation of CRTC1 and 3	(33)
Restraint stress	Rat CRH neurons	Increased expression	(34)
BDNF	Cortical neurons	Increased expression	(35)
Serum stimulation	SCN cells	Increased expression	(36)
Gastrin	Adenocarcinoma cells	Increased expression	(37)
Diet induced obesity	Skeletal muscle	Increased expression	(23)
Metformin	HepG2 cells	Increased expression	(38)

Table 1. List of reported SIK1 stimuli and their effects on SIK1 expression and activity. Many of these stimuli have been confirmed by multiple research teams and in multiple organ systems; only the initial discoveries are listed.

1.2.2 SIK1 substrates and their physiological relevance

Over the years, several SIK1 substrates have been identified with CRTCs and class II HDACs being the most widely studied. Both of these groups of SIK1 targets modulate transcription factor activity (CREB and MEF2) in the nucleus, thus demonstrating SIK1 action as a mechanism for fine-tuning gene expression. Indeed, we most often consider SIK1 as an indirect gene regulator; however, many of the other substrates listed in Table 2 importantly demonstrate that SIK1 regulates proteins in several cellular domains such as the plasma membrane and mitochondria. Additionally, SIK1 phosphorylation of these targets results in a variety of cell signaling and biochemical outcomes that are certainly not limited to changes in gene expression.

Table 2. SIK1 substrates and the effects of phosphorylation			
Substrate	**Phosphorylated amino acid**	**Effect of phosphorylation**	**Citation**
CRTC2	Ser171	Nuclear export; inhibited CREB transcription	(8, 9)
HDA-4 (c. Elegans); HDAC5 (mouse)	Ser259	Nuclear export; de-repression of MEF2 transcription	(10, 11)
Pme1	Ser72	Dissociation with NK complex; increased NK activity	(13, 39)
SREBP-1C	Ser329	Inactivation of SREBP; inhibition of lipogenesis	(40)
p53	Ser15	P53 stabilization; anoikis	(15)
TAU	Thr212, Ser214, Ser356, Ser262	Decreased microtubule assembly	(41)
PDE4D	Ser136*, Ser141	Increased PDE activity; reduced insulin secretion	(22)
P300	undefined	Undefined	(22)
MLTK	undefined	Undefined	(22)
SRF	undefined	Undefined	(22)
M3K3	undefined	Undefined	(22)
MKNK1	undefined	Undefined	(22)
BRAF	undefined	Undefined	(22)
AAKG3	undefined	Undefined	(22)
ARAF	undefined	Undefined	(22)
TAB2	undefined	Undefined	(22)
ZEP3	undefined	Undefined	(22)
RAF1	undefined	Undefined	(22)
ULK1	undefined	Undefined	(22)
NCOR2	undefined	Undefined	(22)
JKIP-1	undefined	Undefined	(22)
SR-B1	Ser496	SR-B1 activation; increased cholesteryl ester uptake	(42)
SMRT	Thr1391	Inhibition of β catenin; decreased metastasis	(43)
MCM2	Ser7, Ser27, Ser41, Y90†, Ser139	Enhanced helicase activity; increased DNA replication	(43)
Table 2. SIK1 substrates reported with kinase assay data. AA refers to rodent sequences. Substrates reported for other SIK family members but not verified in SIK1 are not listed. *dominant AA, †unconventional AA for SIK1-dependent phosphorylation.			

1.3 Myogenesis

1.3.1 Mechanisms and models of muscle regeneration

Skeletal muscle is a remarkably resilient tissue with the ability to regenerate after injury. The regenerative capacity of muscle stems from the ability to reinstate transcriptional programs originally active during embryogenesis. Myogenesis is the formation of muscle tissue through differentiation of muscle progenitor cells (stem cells). Upon activation, muscle precursor cells proliferate, migrate to damaged or developing tissue through chemotaxis, and upregulate the myogenic program (transcriptional cascades critical for inhibition of proliferation and expression of terminal differentiation factors) (44). Common skeletal muscle injury models utilize these well characterized events to study ways in which these processes are regulated and could potentially be harnessed to promote skeletal muscle regeneration in injured patients.

One commonly used model of muscle regeneration involves degeneration of muscle fibers induced by injection of cardiotoxin, a component of snake venom that causes muscle necrosis without disruption of the basal lamina (45). In this model, previously quiescent skeletal muscle progenitor cells are activated upon injury. Peak proliferation of these cells occurs 3 days post injury and repaired muscle fibers can be visualized as early as 5 days post injury. Full recovery of muscle fiber function and size occurs by 21 days after injury. We previously utilized this model to study the function of the transcription factor CREB in the regenerative process because molecules that increase cAMP production and activate downstream signaling have been demonstrated to promote skeletal muscle growth.

1.3.2 CREB regulates skeletal muscle regeneration and muscle maintenance

Briefly, we found that CREB activity promotes skeletal muscle repair through enhanced proliferative capacity of muscle progenitor cells (31). Interestingly, in this study we found that SIK1, a known CREB target gene, was induced in regenerating skeletal muscle. This demonstrated that skeletal muscle injury was yet another stressful stimulus of SIK1 expression but any relevant roles of SIK1 expression during myogenesis remained elusive.

In addition to regulating myogenesis, CREB is important for maintenance of un-injured adult skeletal muscle. Expression of a dominant-negative CREB inhibitor, ACREB, results in severe muscle necrosis which is rescued by re-expression of SIK1 (11). The mechanism of rescue described in this work involved removal of class II HDAC inhibition of MEF2-dependent transcription. Since MEF2 is a critical myogenic factor and SIK1 expression increases in regenerating muscle, we investigated SIK1 action during muscle development. This investigation will be discussed in chapter 3 of this dissertation.

Introductory remarks will conclude with a discussion of glucose metabolism and known regulatory mechanisms of this process. The relevance of this topic became apparent as our investigation of SIK1 loss of function mutant mice progressed (chapter 4). As previously described, SIK1 is a highly regulated protein that is expressed in multiple tissues in response to a wide variety of stimuli that impede homeostasis. Impaired glucose metabolism challenges homeostasis and is regulated by SIK1 (mechanism discussed in chapter 4).

1.4 Metabolic pathology and glucose utilization

1.4.1 Type 2 diabetes is an epidemic

Type 2 diabetes mellitus (T2DM) is a severe metabolic disorder of increasing prevalence affecting 29.1 million Americans in 2012 and contributing to approximately $245 billion per year in health care costs in the United States alone (American Diabetes Association). This disease is characterized by insulin resistance and hyperglycemia and is strongly associated with obesity. Current treatments for T2DM include diet and exercise and a medley of oral medications, biguanides (Metformin) being the most commonly prescribed. Although T2DM medications have consistently evolved since the 1950s, there is still a need for discovery of improved treatments, as currently prescribed medications are often associated with adverse side effects, are contraindicated for patients with common comorbidities, and are burdensome in daily life. In the search for new treatments, much interest has emerged in targeting protein kinases to treat T2DM. This is due to the fact that multiple critical nodes of the insulin signaling pathway are often regulated, positively and negatively, by phosphorylation and kinases are good drug targets that can often be selectively inhibited by synthetic small molecules.

1.4.2 Extracellular insulin action

Glucose is an important fuel source that requires external regulation to maintain blood concentrations within a discrete range (70-100 mg/dL during fasting) which is surprisingly consistent among mammalian species (American Diabetes Association). Tight regulation of blood glucose concentrations is necessary to provide adequate fuel supply to tissues without oversaturation which can cause severe medical issues

including neuropathy, retinopathy, and even death. Insulin is a critical hormone for maintaining mammalian glucose homeostasis. The main function of insulin is to reduce blood glucose concentrations in an environment where fuel supply is ample and blood glucose levels are higher than the demand of tissues. Ineffective insulin action, due to a lack of insulin production in type 1 diabetes or ineffective insulin action in T2DM, can lead to life-threatening hyperglycemia. After meal consumption, insulin is released from pancreatic beta cells and enters the blood stream where it can contact insulin receptors on target tissues.

The ultimate physiological effects of insulin are tissue dependent with an ultimate goal of blood glucose reduction. For instance, in the liver, insulin inhibits *de novo* glucose production (gluconeogenesis) and promotes glucose storage (glycogenesis) (46-48). In skeletal muscle, insulin promotes glucose uptake and conversion to glucose-6-phosphate to irreversibly remove glucose from circulation (49, 50). Additionally, insulin reduces blood glucose concentrations by promoting glucose uptake into adipose tissue and inhibiting fatty acid release which counterbalances glucose uptake into skeletal muscle through the Randle cycle (51). The diverse physiological effects and critical nature of insulin action have inspired multiple researchers to invest significant time and energy into characterizing the precise intracellular signaling networks underlying insulin action. While new advances are regularly made regarding our understanding of insulin signaling and regulation, a canonical insulin signaling pathway has been described.

1.4.3 Intracellular insulin signaling

Insulin target tissues express insulin receptors on their cell surfaces so the circulating hormone can activate intracellular signaling. There are two mammalian isoforms of the insulin receptor which are receptor tyrosine kinases (52). Insulin bound receptors undergo auto-phosphorylation on conserved tyrosine residues (Y1162, Y1163) (53) which in turn promotes recruitment of adaptor proteins such as insulin receptor substrate proteins (IRS). Notably, insulin receptors can also be activated by insulin-like growth factors (IGF-1 and IGF-11). Recruitment of IRS proteins (through IR dependent tyrosine phosphorylation) then exposes binding sites for downstream effectors, most notably phosphoinositide 3-OH kinase (PI3K) which catalyzes the conversion of phospholipids in the plasma membrane (PIP_2 to PIP_3). The end product of this conversion, PIP_3, then activates the serine/threonine kinase Protein kinase B (AKT) through membrane recruitment. This positions AKT in close proximity to 3-phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates and activates AKT at T308 (54, 55). Subsequent phosphorylation of Ser473 leads to full activation of AKT and stimulation of downstream effectors.

AKT is considered a critical node of the intracellular insulin signaling pathway and is often used as a read out of the integrity and effectiveness of insulin action in tissues (56, 57). One pathway downstream of AKT activation involves AKT-dependent activation of vesicle-associated proteins (AS160 and Rab) which regulate vesicle fusion with the plasma membrane and glucose transporter (GLUT) incorporation (58-60). GLUT proteins facilitate passive diffusion of glucose down the extracellular/intracellular concentration gradient and vary in sensitivity to insulin dependent membrane

incorporation. Some GLUT proteins are endogenously located within the plasma membrane and are considered insulin insensitive. Such glucose transporters always facilitate glucose diffusion (i.e. GLUT1) into cells. Conversely, other GLUT isoforms are stored in cytoplasmic vesicles and do not facilitate glucose import until they are incorporated into the plasma membrane in response to (61, 62) stimulation by insulin or exercise (i.e. GLUT4) (63). Quantification of membrane bound insulin-sensitive GLUT isoforms serves as a useful tool for determining the ultimate effectiveness of intracellular insulin signaling and correlates with the ability of peripheral cell types to remove glucose from circulation.

1.4.4 Mechanisms of insulin resistance

Diet-induced obesity is a prevalent issue in modern society and is associated with T2DM. Insulin resistance partly characterizes this disorder. After continued overnutrition and persistent hyperglycemia, tissues eventually become resistant to the insulin actions described above. Our current mechanistic understanding of insulin resistance involves aberrant accumulation of fatty acid derivatives (Acyl-CoAs and diacylglycerol, DAG, for example) in skeletal muscles of obese patients. Oversupply of fats and carbohydrates contributes to these defects by altering the balance of glucose and fatty acid metabolism. High concentrations of plasma free fatty acids, as would occur in the obese state, impair glucose uptake into skeletal muscle. Reduced glucose availability for fueling skeletal muscle ATP synthesis, increased transcription of enzymes involved in β-oxidation, and oversupply of fatty acids leads increased fatty acid oxidation to meet muscle fuel demands (64, 65), which further reduces glucose oxidation. Continued overnutrition leads to such elevated fatty acid availability that even

hyperactive β-oxidation cannot utilize all available substrates. This leads to aberrant accumulation of lipids. Accumulation of plasma membrane bound fatty acids has been shown to activate Protein kinase C θ (PKC-θ), a known inhibitor of IRS activation and PI3K activity (66-68). In other models, increased DAG has been shown to inhibit insulin action through a mechanism independent of the canonical insulin signaling pathway. Oversupply of fatty acids has been suggested to impair mitochondrial function through overloading the organelles and stressing respiratory capacity (69-71). This latter effect has been demonstrated to impair insulin dependent glucose uptake independently of the canonical insulin signaling pathway, although the exact molecular mechanism(s) responsible are still elusive; therefore, it is clear that insulin sensitivity relies on more than an intact canonical insulin signaling pathway.

A relatively new field of study in the realm of insulin resistance involves analysis of mitochondrial health, organization, and quality control in the etiology of this disorder. An interesting correlation between mitochondrial hyper-fission and obesity has been described (72). Hyper-fragmentation of skeletal muscle mitochondria are thought to be less efficient power generators than fused mitochondria but further studies are needed to confirm this notion (73). Interestingly, the mitochondrial quality control protein, Parkin, has been described as a critical component for maintenance of insulin sensitivity in skeletal muscle cells (74) suggesting that healthy mitochondria are an integral component of insulin action.

Chapter 2: Materials and methods

2.1 Isolation of primary skeletal muscle cells. Forelimb and hindlimb muscles were collected from mouse neonates (p0-p3), washed in 1% glucose/ PBS and digested in type II collagenase (2500 U/mL, Worthington CLS2) at 37ºC for 3-5x 12 minute digestions (until all muscles were digested). Excess debris was then separated from the desired cell population through filtration (40μm, BD Falcon #352340). Cells were then allowed to adhere to Matrigel-coated tissue culture dishes (BD Biosciences) overnight in DMEM containing 20% FBS, 25% 2xF-10 (Sigma N6635), 1% penicillin/streptomycin/fungizone, and 2.5 ng/mL basic fetal growth factor (bFGF; Peprotech #450-33) supplementation. For differentiation time courses and myotube experiments, primary cells were differentiated on Matrigel-coated dishes or glass coverslips for up to 7 days in DMEM media containing 2% horse serum (heat inactivated) and 1% penicillin/streptomycin/fungizone. Cells were monitored daily and media was replaced every other day.

2.2 Diet-induced obesity modeling. Male mice (10-12 weeks old) were weighed prior to high fat diet feeding and subsequently weighed weekly or bi-weekly over the course of high fat diet feeding (Test Diet #0058170; 60% energy from fat). Subjects were housed with 1-3 mice per cage to ensure dominance hierarchy over food consumption was avoided and every mouse gained weight. Weight gain among genotypes was compared using student's t-test. Food consumption on singly housed mice was initially measured on a daily basis for one week and no difference in amounts consumed was observed.

2.3 Metabolic testing.

Glucose tolerance test. Mice were fasted overnight (16 hours) prior to the experiment. Body weights and fasting blood glucose measurements were taken from tail (OneTouch ultra mini glucometer system). Glucose was then injected intraperitoneally (0.75 U/kg for lean mice; 1.5 U/kg for obese mice) and blood glucose measurements were collected every 15-30 minutes through the course of 160 minutes total.

Hyperinsulinemic-euglycemic clamp. Mice were fed high fat diet for 16-18 weeks and were transferred to Baylor College of Medicine Mouse Metabolism Core where the experiment was conducted by our collaborator Dr. Pradip Saha. Briefly, mice were given 3-5 days to acclimate to the new environment and were then fasted overnight (16 hours) prior to the experiment. Mice remained unrestrained while infused with a super-physiological dose of insulin (Humulin R, 8mU/min/kg body weight) and a variable dose of cold glucose to counterbalance insulin effects and clamp blood glucose levels to a range of 100-140 mg/dL. Once dynamic steady state was achieved, blood glucose levels were measured and hepatic glucose output and glucose infusion rate were calculated.

Indirect Calorimetry. Mice were acclimated to single-house calorimetry chambers (Omnitech Electronics Inc. Fusion v5.0 PhysioScan edition) for 3-5 days prior to experimentation. Oxygen consumption and heat production were measured over 3 dark cycles and were used to calculate VO_2, $VC0_2$, and RER. Total activity was simultaneously measured using infrared activity frames (Kinder Scientific) that fit around the calorimetry chambers.

2.4 Biochemical analysis of signaling pathways. Lysates were made from mouse tissue or primary skeletal muscle cells with a modified RIPA buffer supplemented with protease inhibitors (benzamidine-HCl (1.6 μg/mL), phenanthroline (1 μg/mL), aprotinin (1 μg/mL), leupeptin (1 μg/mL), pepstatin A (1 μg/mL)), sodium vanadate (1mM), AEBSF (100μM), and MG-132 (10μM). The following antibodies were used for western blotting: SIK1 (in house custom antibody; 1:1000 overnight), Parkin (Cell Signaling # 4211; 1:1000 overnight), pAKT Ser473 (Cell Signaling #4060, 1:1000 overnight), pAKT Thr308 (13038), AKT2 (Cell Signaling # 5239S, 1:200 overnight for IP, 1:5000 overnight for western blots), pMFF (Cell Signaling # 49281, 1:1000 overnight), total MFF (Proteintech # 17090-1-AP, 1:1000 overnight), pIRS1 (Cell Signaling # 2389, 1:1000 overnight), total IRS1 (Cell Signaling # 3407, 1:1000 overnight), phospho HDAC 4/5/7 (Cell Signaling # 3443, 1:5000 overnight), HDAC4 (Cell Signaling # 2072, 1:1000 overnight), HDAC5 (Cell Signaling # 2082S, 1:1000 overnight), pCREB (Cell Signaling # 9198s, 1:5000 overnight), CREB (Cell Signaling # 9197, 1:1000 1 hour), CRTC1, 2, and 3 (Cell signaling #s 2587, 3826s, 2720s), IRβ (Cell Signaling # 3025, 1:1000 overnight), MEF2 (Cell Signaling # 5030, 1:1000 overnight), HSP90 (SantaCruz, 1:5000 1 hour), ATP5b (SantaCruz #SC-55597, 1:2000 1 hour).

2.5 Glucose transporter staining in mouse skeletal muscle. Quadriceps muscles were mounted in OCT and cut in cross-section (7μm thick) such that samples for staining were collected from the belly of the muscle (the area of largest diameter). Tissue sections were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum, and incubated in a humidified chamber overnight with anti GLUT4 (Millipore #CBL243, 1:300) or anti GLUT1 (SantaCruz #SC377228, 1:300) primary antibodies.

After a 1x PBS wash, secondary antibodies (anti-rabbit Alexa 549, 1:200) were introduced for 1 hour. Sections were then washed 3 times in PBS and coverslips were mounted with 4% n-propyl gallate and nail polish to seal. Samples were imaged using a Nikon A1R confocal microscope and the number of fibers with positive GLUT staining on myofiber plasma membranes were quantified.

2.6 Mitochondrial isolation from mouse skeletal muscle. Skeletal muscles (quadriceps) were collected from mice anesthetized with isoflurane and mitochondrial isolations were performed as previously described by others (75). Briefly, muscles were immediately mulched and pulverized in glass dounces with Teflon plungers in a Tris/sucrose/EDTA isolation buffer with protease inhibitors and N-Ethylmaleimide (5mM). Mitochondria were separated from the rest of the muscular contents through percoll gradient (5%, 26%, 40% stacked layers) centrifugation. Once mitochondria were collected from gradients (from the 26%-40% interface), they were washed in excess isolation buffer and lysed in 8M urea buffer (used as a 2x stock) or fixed on EM grids for negative staining with NanoVan for morphological analysis (protocol developed by Dr. Neal Waxham).

2.7 Gene expression analysis. Gene expression patterns were quantified in Cre-recombinase (control) and SIK1-MKO (*Myf5*-Cre) quadriceps muscles collected from obese male mice. Messenger RNA samples were extracted from tissue lysates by phenol-chloroform purification. Reverse transcriptase PCR was then performed using oligo dT-20 and M-MLV enzyme to create cDNA for subsequent microarray analysis in collaboration with Dr. Tuan Tran (Quantitative Genomics and Microarray Core Lab at UT Health McGovern Medical School). Gene expression levels were compared among

genotypes and differences in select target gene expression levels were confirmed through quantitative PCR analysis using the same cDNA inputs and unique target gene primers from those used in the array. Additional QPCR analyses were conducted for targets of interest using the primers listed in Table 3.

Table 3. Primer list for mRNA measurements by Quantitative PCR

Target	Forward primer sequence	Reverse primer sequence
Cox7a	CAGCGTCATGGTCAGTCTGT	AGAAAACCGTGTGGCAGAGA
Crtc2	CCACCAGAACTTGACCCACT	GGCTGCTGCAATCTCCTTAG
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Glut1	CTCTGTCGGGGGCATGATTG	TTGGAGAAGCCCATAAGCACA
Glut4	CCAGCCTACGCCACCATAG	TTCCAGCAGCAGCAGAGC
Glut12	GCCAGCTTGCTTGTTTATGT	GCTGTGTTGGCACTAATTCTTCCTG
G6Pase	TGCTGTGTCTGGTAGGCAAC	AACATCGGAGTGACCTTTGG
Hdac5	AAGGATGAGGATGGCGAGAGTG	CCAGGAGCAGCAGGTGAGG
L32	TTAAGCGAAACTGGCGGAAAC	TTGTTGCTCCCATAACCGATG
Mef2a	AACCCAGGGAGTTCACTCGT	CATGCTCGAATCTGCTAATGTTG
Mef2b	TTTCACCAAGCGCAAGTTCG	GTCGCAGTCACAAAGCACG
Mef2c	ACGAGGATAATGGATGAGCGT	ATCAGTGCAATCTCACAGTCG
Mef2d	CAGGCGCTATGGGTCATCTG	GCTACTTGGATTGCTGAACTGC
mtCox2	CAGTCCCCTCCCTAGGACTT	TCAGAGCATTGGCCATAGAA
Nr4a2	CGCCGAAATCGTTGTCAGTA	CGACCTCTCCGGCCTTTTA
Nr4a3	TCAGCCTTTTTGGAGCTGT	TGAAGTCGATGCAGGACAAG
Park2	AAACCGGATGAGTGGTGAGT	AGCTACCGACGTGTCCTTGT
Pdh1b	AAGAGGCGTTTTCACCGCTC	GTCACCGTATTTCTTCCACAGG
Pdk4	AGGGAGGTCGAGCTGTTCTC	GGAGTGTTCACTAAGCGGTCA
Pepck	AGAGTCACCCCTTCCCACTC	CCCTAGCCTGTTCTCTGTGC
Pgc1a	GGACGGAAGCAATTTTTCAA	TTACCTGCGCAAGCTTCTCT
Sdh	TGGTGGAACGGAGACAAG	CAGCGGTAGACAGAGAAGG
SIK1 (3’ end)	ATTGTCCCCATGTTTGTGGT	TACTGCTGCGGTGAGATTTG
SIK1 (5’ end, kinase domain)	GGAGGTCCAGCTCATGAAAC	CTGCCTAGCCTCGTTTTCAC
SIK2	TTGATGGACCAACTCTCCCTAT	TTGGAAGGATCTAGGACCAACA
SIK3	CTCAAGCACACTGACCAAAGG	GGCCTGACTCACAGTTCCC

Table 3. Mouse sequences used for QPCR measurements

2.8 Proteome analysis. Protein composition was analyzed in mitochondria isolated from wild type and global SIK1-KO mouse skeletal muscle (quadriceps) from obese male mice. Mice were fed high fat diet for 14 weeks and tissue was collected under isoflurane anesthesia. Mitochondria were isolated as described above and protein lysates were made by diluting samples with an 8M urea, 4% SDS buffer (used as a 2x concentrated buffer). Samples were run into 1cm of a 7.5% acrylamide gel and subsequently analyzed by mass spectroscopy in collaboration with Dr. Li Li (Clinical and Translational Proteomics Service Center at the Institute for Molecular Medicine). Proteins of interest were identified by first eliminating non-mitochondrial proteins known to be abundant skeletal muscle structural proteins (e.g. Myosin heavy chains). Then, proteins were eliminated as background when they did not reach a minimum threshold score of 2 for matches or number of sequences identified. The emPAI scores of the remaining targets were analyzed for each mouse and overall abundance among genotypes was compared. Additionally, post-translational modifications including oxidation (M), acetylation (N-term), phosphorylation (S/T or Y), and glycosylation were analyzed. No notable alterations in post translational modifications were identified between genotypes.

2.9 Structural and mitochondrial analysis of mouse skeletal muscle. Skeletal muscles (quadriceps, soleus, and extensor digitalis longus) from lean and obese wild type and SIK1-KO male mice were collected under isoflurane anesthesia and immediately fixed in 3% TEM grade glutaraldehyde. In collaboration with Integrated Microscopy Core at Baylor College of Medicine and CV Pathology Core at Texas Heart Institute, samples were cut in cross-section (60-80 nm) and contrast stained with uranyl

acetate followed by lead citrate. Skeletal muscle structure and mitochondrial composition were analyzed using transmission electron microscopy (TEM) on a JEOL JEM-1400 transmission EM. Images were collected by an experimenter blinded to genotype. Consistent parallel alignment of skeletal muscle Z-lines with perpendicular sarcoplasmic reticulum structures was a requirement for all images used to quantify mitochondrial content and size to ensure mitochondrial networks were viewed from similar vantage points. Mitochondrial area was analyzed from .dm4 images using FIJI software. After all measurements were collected, blinded samples were decoded and mitochondrial areas were compared amongst genotypes.

Chapter 3: Regulation of SIK1 abundance and stability in muscle

3.1 Rationale for studying SIK1 in skeletal muscle

Skeletal muscle is a remarkably adaptable organ with the ability to reinstate developmental programs after insult. This response to injury enables re-establishment of complex structural networks and metabolic processes characteristic of healthy skeletal muscle. For instance, it is well documented that mouse skeletal muscle has the capacity to regenerate after chemical-induced injury or genetic manipulation of structural proteins (reviewed in (76)). We previously demonstrated that SIK1 expression increases during myogenic repair of cardiotoxin injured skeletal muscles and in mice afflicted with muscular dystrophy (31). Since we observed increased SIK1 expression during myogenesis *in vivo* (31) and ectopic expression of SIK1 in adult skeletal muscle has a profound protective effect against muscle necrosis (discussed in chapter 1; (11)), we hypothesized that SIK1 is a critical regulator of myogenic differentiation of muscle precursor cells. To test this hypothesis, we used a common muscle cell line (C2C12) and isolated muscle precursor cells from neonatal mice. We then characterized SIK1 expression and regulation in these cells and in response to manipulation using cAMP pathway agonists and RNAi for loss of function analyses during myogenic differentiation (21). Through this study, we not only uncovered an important role for SIK1 in myogenic differentiation, but also described important regulatory elements for SIK1 action in muscle cells that have the potential to further inform us on how to control this protein in possible clinical settings.

3.2 Post-transcriptional regulation of SIK1

We found that SIK1 protein levels are nearly undetectable in undifferentiated cultured muscle precursor cells (Fig 1, lane 1). Moreover, SIK1 protein levels are only transiently increased in undifferentiated muscle precursor cells after incubation with forskolin/IBMX to stimulate cAMP production and downstream PKA/CREB signaling. We included cycloheximide (CHX) treatment in these experiments to prevent new protein synthesis and gauge the true half-life of SIK1 protein. We hypothesized that the transient nature of this protein in undifferentiated cells was due to protein degradation after we observed no changes in SIK1 mRNA stability after forskolin treatment (Fig 2). We further demonstrated that SIK1 is post-translationally modified via ubiquitination by a yet unidentified E3 ligase in isolated muscle precursor cells. Our further characterization of SIK1 in muscle progenitor cells yielded results demonstrating SIK1 protein stabilization in the presence of MG-132 (proteasome inhibitor) and destabilization of nuclear-localized SIK1 (Fig 1). These results contributed to our understanding of the tight regulation of SIK1 in muscle cells and prompted us to investigate how upstream cAMP signaling may play a role in this regulation.

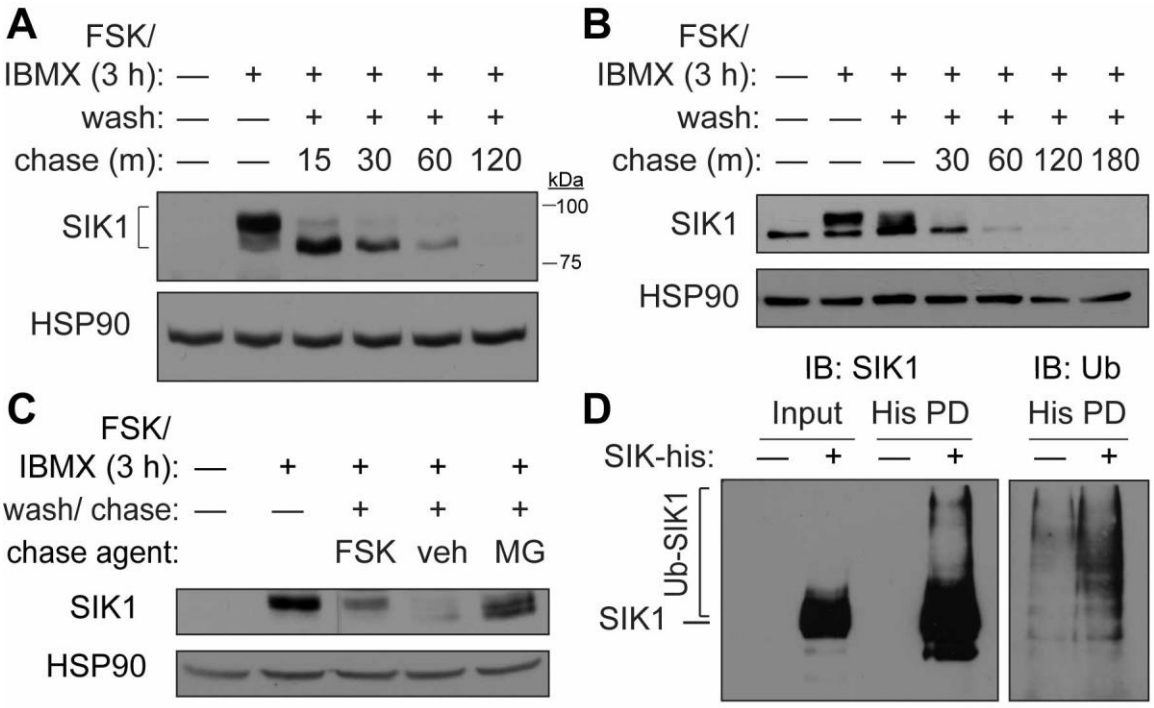

Figure 1. SIK1 protein is degraded by the proteasome in myoblasts. (A) Endogenous SIK1 and HSP90 proteins in C2C12 myoblasts treated with FSK/IBMX (3h) to induce SIK1 protein, washed, and chased in medium containing CHX (n = 8). (B) Endogenous SIK1 protein in primary mouse skeletal myoblasts following 3h of FSK/IBMX priming and CHX chase (n = 5). (C) Endogenous SIK1 protein in C2C12 myoblasts treated with FSK/IBMX (3h) and chased in medium containing the indicated agent (FSK, DMSO vehicle, or MG-132 but no CHX) for 30 min (n = 7). MG-132 was added 30 min before wash and chase. (D) SIK1 ubiquitylation visualized by pull-down of his-tagged SIK1 under denaturing conditions. Western blots show SIK1 (Left) and ubiquitin (Right) in input and pull-downs (His PD) (n = 3). Dr. Dmitry Akhmedov performed experiments for panels C and D. Published in PNAS and used with journal and co-author permission: Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R: Regulation of SIK1 abundance and stability is critical for myogenesis. Proceedings of the National Academy of Sciences of the United States of America 2013, 110(1):117-122.

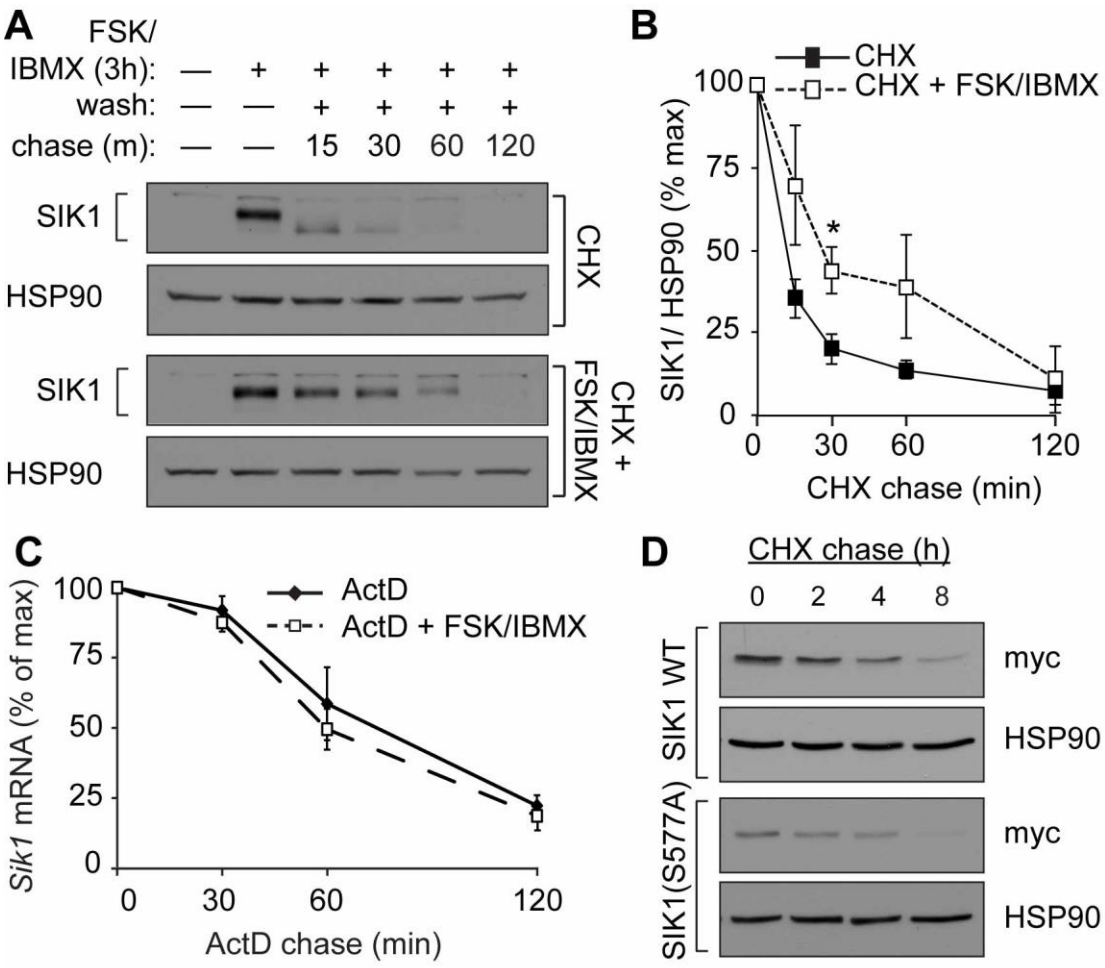

Figure 2. SIK1 degradation is regulated by cAMP signaling. (A) SIK1 protein in C2C12 cells primed with FSK/IBMX for 3h and chased in medium with CHX ± FSK/IBMX (n = 4). (B) Quantification of data in A. Half-life of endogenous SIK1 (n = 3, mean percent SIK1 remaining normalized to HSP90 ± SD). (C) *Sik1* mRNA degradation in C2C12 cells stimulated for 3h with FSK/IBMX, chased with ActD ±FSK/IBMX, expressed as normalized percent of maximum ± SD (average of n = 2, representative of n = 4). (D) CHX chase assays of myc-SIK1 (WT or S577A) in C2C12 cells. Dr. Dmitry Akhmedov performed experiments for panel D. Published in PNAS and used with journal and co-author permission: Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R: Regulation of SIK1 abundance and stability is critical for myogenesis. Proceedings of the National Academy of Sciences of the United States of America 2013, 110(1):117-122.

Phosphorylation of SIK1 by PKA on S577 had previously been demonstrated to cause nuclear export of SIK1 thus preventing phosphorylation of SIK1 nuclear targets (77). Importantly, it was also shown that this phosphorylation does not affect overall

catalytic ability of SIK1 to phosphorylate substrates. Translocation of SIK1 appears to merely serve as an influence toward catalytic activity on a different subset of SIK1 substrates. For example, SIK1 has separate effectors in nuclear and cytoplasmic compartments (discussed in chapter 1 and 5). In addition to this previously characterized PKA phosphorylation site, we identified another PKA phosphorylation site at T475 based on the common PKA target motif (RRHTL) conserved within the SIK sequences in multiple species (Fig 3A,B). Indeed, *in vitro* kinase assays with recombinant PKA and WT SIK1 or T475A (non-phosphorylatable mutant) confirmed PKA-dependent phosphorylation at this site (Fig 3D). Additionally, we found that T475 phosphorylated SIK1, or phospho-mimetic mutant T475E, displayed enhanced protein stability and altered degrees of ubiquitination (Fig 3E-G). This finding supports the idea that PKA phosphorylation of SIK1 not only redirects the kinase to new cellular compartments to interact with cytoplasmic targets, but also enhances the half-life of SIK1 protein in muscle precursor cells. Interestingly, threonine at the 475 position proved to be a critical amino acid for proteasome degradation of SIK1 because mutation to any other amino acid resulted in enhanced stabilization of the kinase. Manipulation of this site may be a valuable tool in future drug development as a mechanism for enhancing or destabilizing this kinase.

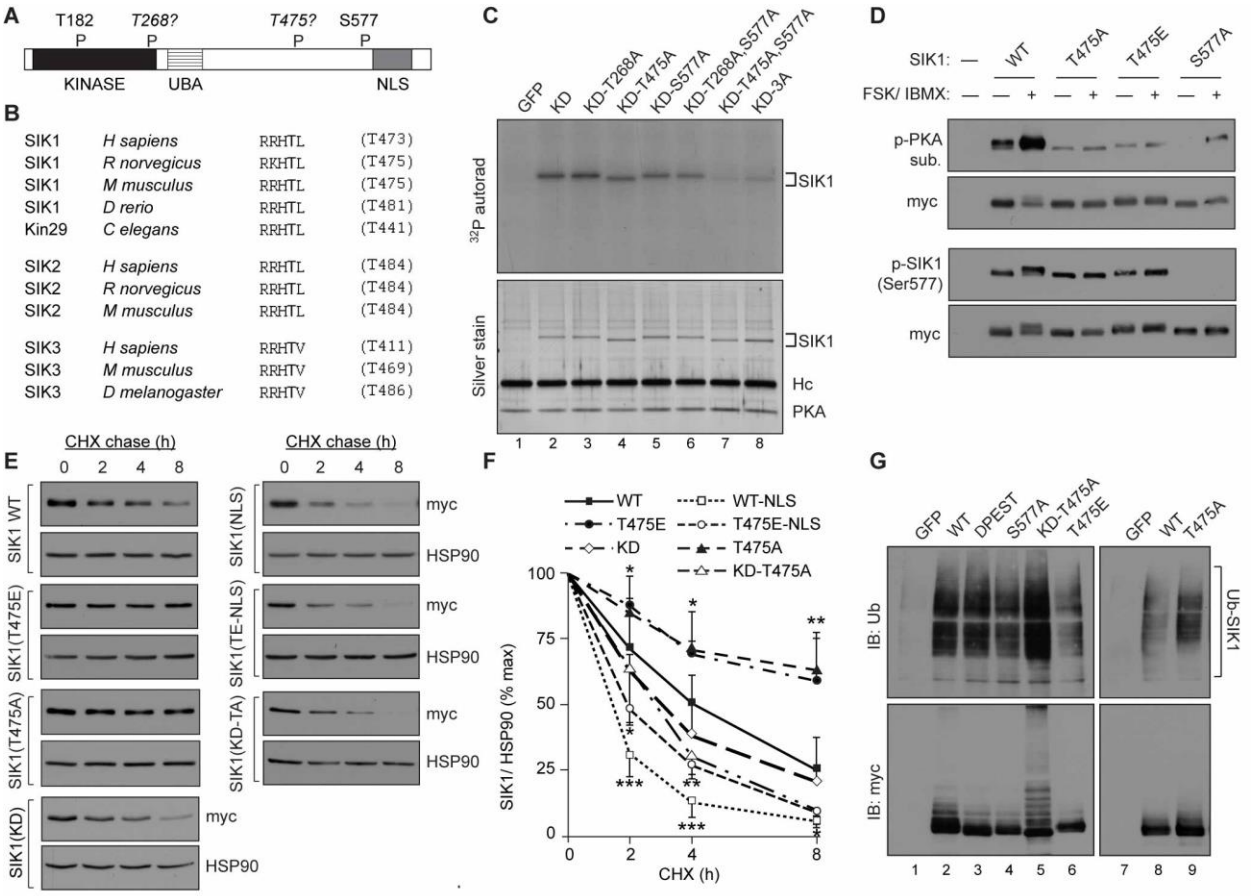

Figure 3. SIK1 phosphorylation by PKA on T475 contributes to stability. (A) Diagram of SIK1 protein with known (T182 by LKB1; S577 by PKA) and hypothesized (italic) phosphorylation sites. NLS, nuclear localization signal; UBA, ubiquitin-associated domain. (B) Sequence surrounding T475 in SIK orthologs. (C) *In vitro* kinase assay of recombinant PKA on purified SIK1 kinase-inactivated (KD) mutants. KD-3A, SIK1(KD-T268A,T475A,S577A). Upper, ^{32}P autoradiogram; Lower, silver stain. SIK1, IgG heavy chain (Hc), and PKA are indicated (n = 3). (D) Western blots of immunoprecipitated myc-SIK1 mutants with phospho-PKA substrate, phospho-SIK1(S577), and myc antibodies. HEK293T cells were pretreated with vehicle or FSK/IBMX (30 min) (n = 3). (E) CHX chases of SIK1 mutants in C2C12 cells (n ≥ 3 per mutant). (F) Quantification of data in E (mean of n ≥ 3 ± SD); significance to WT shown for T475E, WT-NLS, and TE-NLS. (G) Ubiquitylation of SIK1 mutants from MG-132 treated HEK293T cells purified by his-SIK pull-down under denaturing conditions, probed for ubiquitin or SIK1 (n = 3). Dr. Dmitry Akhmedov performed experiments for these panels. Published in PNAS and used with journal and co-author permission: Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R: Regulation of SIK1 abundance and stability is critical for myogenesis. Proceedings of the National Academy of Sciences of the United States of America 2013, 110(1):117-122.

We were interested in investigating a previously described PEST domain identified in the SIK1 sequence because it lies adjacent to T475 and many proteins possessing PEST domains are controlled by proteasome degradation. This proves to be true for SIK1 as well. Although we did not detect changes in SIK1 ubiquitination when the PEST domain was mutated, we did find that SIK1 mutants lacking the PEST domain (ΔPEST), were more stable than wildtype SIK1 (Fig 4). Together, the characteristics of SIK1 domains and phosphorylation sites reveal tight regulation of SIK1 in undifferentiated muscle cells that seems to favor very limited action of this kinase both in cellular localization and in time. It will be interesting in future studies to determine the reason cAMP/PKA signaling favors enhanced SIK1 half-life with concurrent cytoplasmic localization. Since we knew SIK1 to be critical for myocyte enhancer factor-2 (MEF2) activity and maintenance of healthy fully differentiated muscle cells, we wondered if SIK1 stability and activity were enhanced with differentiation.

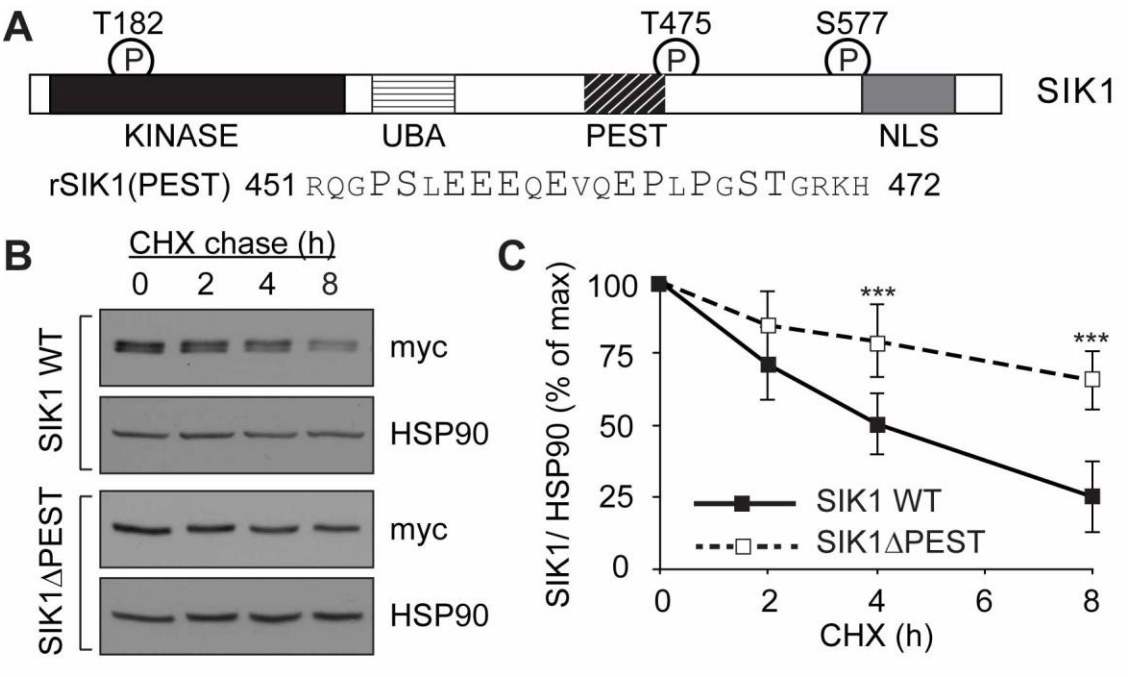

Figure 4. SIK1ΔPEST is a stabilized mutant. (A) Diagram of SIK1 protein showing the PEST domain (AA 451–472) and confirmed phosphorylation sites. PEST sequence from rat SIK1 is shown. (B) CHX chase of SIK1ΔPEST in C2C12 myoblasts (n = 5). (C) Quantification of data in B (mean of n ≥ 5 ± SD). Dr. Dmitry Akhmedov performed experiments for these panels. Published in PNAS and used with journal and co-author permission: Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R: Regulation of SIK1 abundance and stability is critical for myogenesis. Proceedings of the National Academy of Sciences of the United States of America 2013, 110(1):117-122.

We hypothesized that SIK1 abundance and activity would be more critical in differentiated muscle cells compared to muscle precursor cells, so we measured SIK1 half-life in both cell types (both originating from the same isolated primary mouse muscle cells). We found that like progenitor cells, forskolin stimulated SIK1 expression in fully differentiated, contractile, myotubes; however, SIK1 stability in myotubes appeared to be significantly enhanced compared to SIK1 stability in undifferentiated cells. The half-life for SIK1 in forskolin treated myotubes was approximately 2 hours and only about 30 minutes in undifferentiated cells from the same isolations. In line with these results, we found that SIK1 protein expression in myotubes precedes increased

Sik1 mRNA levels (Fig 5A-D). Specifically, SIK1 protein levels dramatically increase after 1 day of differentiation but it takes 3-5 days for *Sik1* mRNA levels to increase to the same degree. This supports the finding that SIK1 protein is stabilized in differentiated myotubes. We reasoned that enhanced SIK1 protein stability likely occurs with differentiation because SIK1 is important for up-regulation of the myogenic program, which was also supported by previous results in the ACREB model. Therefore, we employed a loss of function strategy for determining whether SIK1 is required for myogenic differentiation and/or myotube survival.

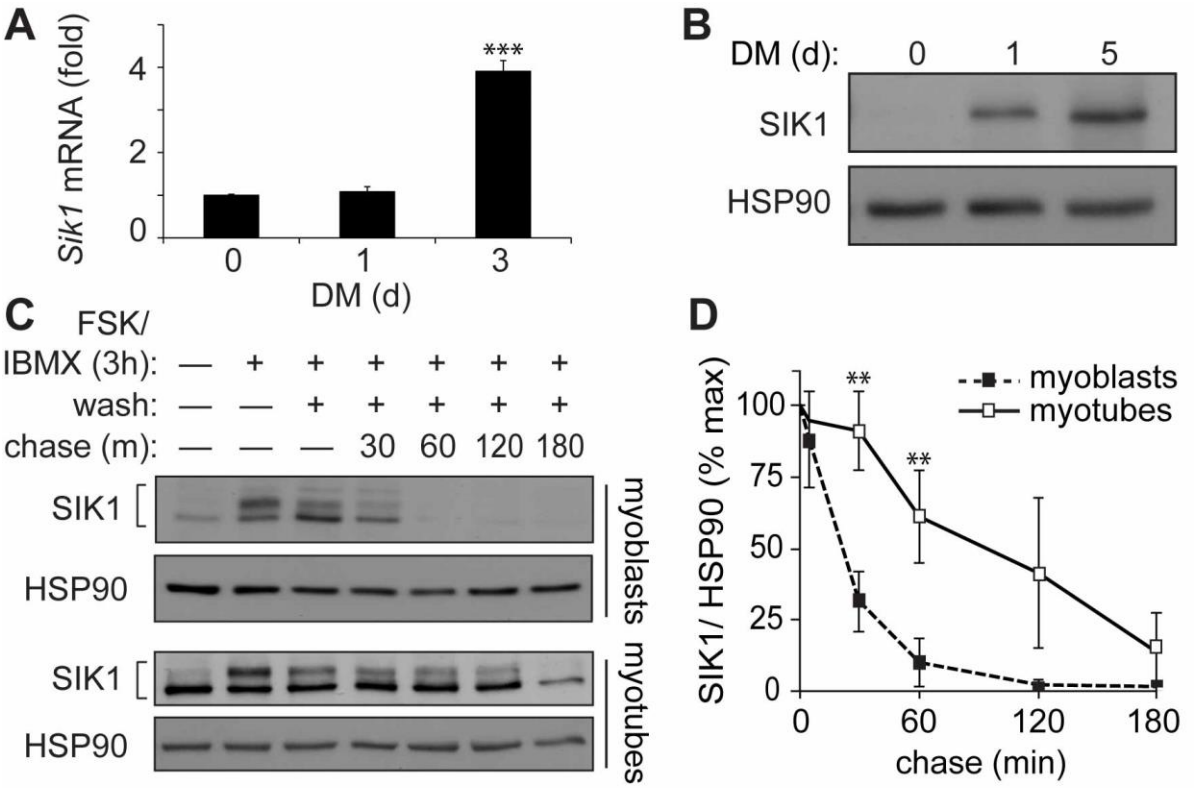

Figure 5. SIK1 protein is stabilized with cellular differentiation. (A) *Sik1* mRNA in primary mouse muscle precursor cells cultured in differentiation medium (DM) for the indicated time in days. *Sik1* mRNA normalized to *Gapdh*, expressed as fold change over day 0. Mean of n = 3 ± SD. (B) SIK1 protein in primary myoblasts incubated in DM for 0–5 d. (C) SIK1 half-life after FSK/IBMX priming and CHX chase in primary myoblasts (upper) versus differentiated primary myotubes (>4 d, Lower). Cells were treated for 3 h with FSK/IBMX, washed, and incubated in medium containing CHX as indicated. (D) Quantification of data in C (mean of three experiments ± SD). Adapted from a figure published in PNAS and used with journal permission: Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R: Regulation of SIK1 abundance and stability is critical for myogenesis. Proceedings of the National Academy of Sciences of the United States of America 2013, 110(1):117-122.

3.3 Myogenesis in SIK1 depleted primary muscle cells

We obtained adenoviruses expressing shRNAs encoding an unspecific sequence (control) or SIK1 targeted sequence to remove SIK1 during the differentiation process

(78). After we confirmed efficient knockdown of SIK1 in SIK1 RNAi treated cultures (SIK1i), we differentiated control and SIK1i cells for 0, 18, or 48 hours and collected whole cell lysates for western blot analysis. Strikingly, 20-30% of cells lacking SIK1 failed to survive depletion of this kinase. Additionally, we found that the remaining surviving SIK1i cells failed to differentiate into contractile myotubes based on gross morphological analysis and failure to adequately upregulate vital myogenic factors including MEF2A and myosin heavy chain (MHC) (Fig 6A-B). We also investigated the degree of class II HDAC phosphorylation in SIK1i cells since we had previously demonstrated this to be an important SIK1 target capable of regulating MEF dependent transcription. Interestingly, class II HDAC phosphorylation was reduced but not completely abolished in SIK1i treated cells (Fig 6A). This supports the idea that additional muscle kinases may regulate class II HDAC phosphorylation during myogenic differentiation but are not sufficient to drive differentiation without the action of SIK1.

Loss of SIK1 in cultured mouse muscle progenitor cells profoundly impairs their ability to form fully functional and healthy myotubes. Our findings from these initial studies reveal a relatively straightforward mechanism by which this could also occur *in vivo* (through inhibition of critical myogenic factors and structural proteins). This model inspired us to imagine SIK1 as a supplement for patients suffering from muscle diseases including dystrophies that could benefit from enhanced myogenesis for repair of damaged muscle tissue. For this reason, we invested in creating the first conditional SIK1 knockout mouse line (23) with the intention of revealing skeletal muscle defects in SIK1-KO and muscle specific SIK1 knockout mouse lines. The phenotypes we

uncovered in the follow up studies were both surprising and intriguing and expanded our research genre as SIK1 is clearly a protein with several diverse roles in skeletal muscle.

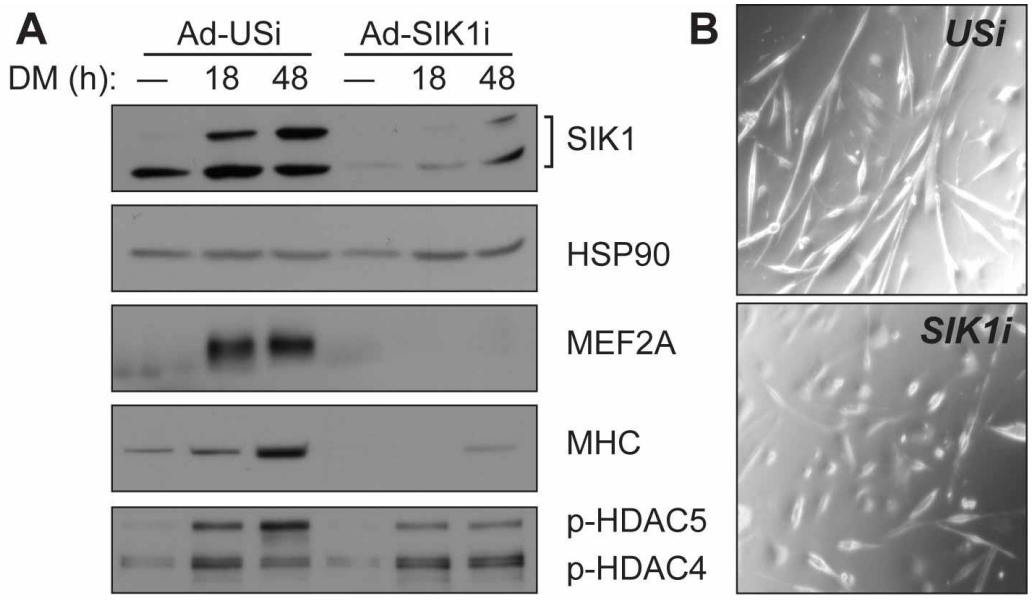

Figure 6. SIK1 is required for differentiation of primary mouse myoblasts *in vitro*. (A) Western blots of differentiation markers during primary myoblast differentiation (hours in DM) after infection with Ad-USi or Ad-SIK1i. (B) Phase-contrast images of infected myoblasts 48 h after differentiation. A and B represent three experiments. Adapted from a figure published in PNAS and used with journal permission: Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R: Regulation of SIK1 abundance and stability is critical for myogenesis. Proceedings of the National Academy of Sciences of the United States of America 2013, 110(1):117-122.

Chapter 4: Skeletal muscle SIK1 is a metabolic regulator

4.1 Rationale for studying SIK1 in mice and generation of SIK1 conditional knockout lines

We previously demonstrated that SIK1 expression and stability increase during the development of contractile muscle cells and is critical for myogenesis *in vitro*. Cultured muscle progenitor cells lacking SIK1 fail to fully upregulate the myogenic transcription factor MEF2a and the structural protein myosin heavy chain (MHC). While cultured mouse muscle progenitor cells are a valuable tool for testing cell signaling pathways, and are easily manipulated with drug treatments and RNAi adenovirus infections, they do not fully represent muscle stem cells in their original niche. Confirmation of signaling events and phenotypes in animal models are more powerful mechanisms for positioning new protein targets as potential therapeutics for future use in humans.

We were interested in determining the effects of SIK1 depletion in muscle stem cells *in vivo* because we reasoned that ablation of SIK1 might impair muscle development or could result in lethality since loss of critical myogenic factors such as MEF2C, Myogenin and Myf5/MyoD (double knockout) are not consistent with viability (79-81). To address these possibilities, we harnessed the power of Cre-*LoxP* technology (82, 83) to create the first SIK1 conditional knockout mouse line (23). We inserted *LoxP* sites into either side of the exons encoding the SIK1 kinase domain. This created a flank of exons 2-7 which could then be excised from the rest of the gene when expressed in the presence of Cre recombinase (Fig 7). We then generated global SIK1 knockout mice using a germline Cre (*GDF9*-Cre used to delete exons encoding the SIK1 kinase domain in all cells including germline haploids) and tissue specific knockout

mouse lines using Cre recombinase linked to promoters expressed solely in tissues of interest (*Myf5*-Cre for muscle and brown fat, *Albumin*-Cre for liver, *Adiponectin*-Cre for white and brown fat, and *Nestin*-Cre for deletion of SIK1 in the brain).

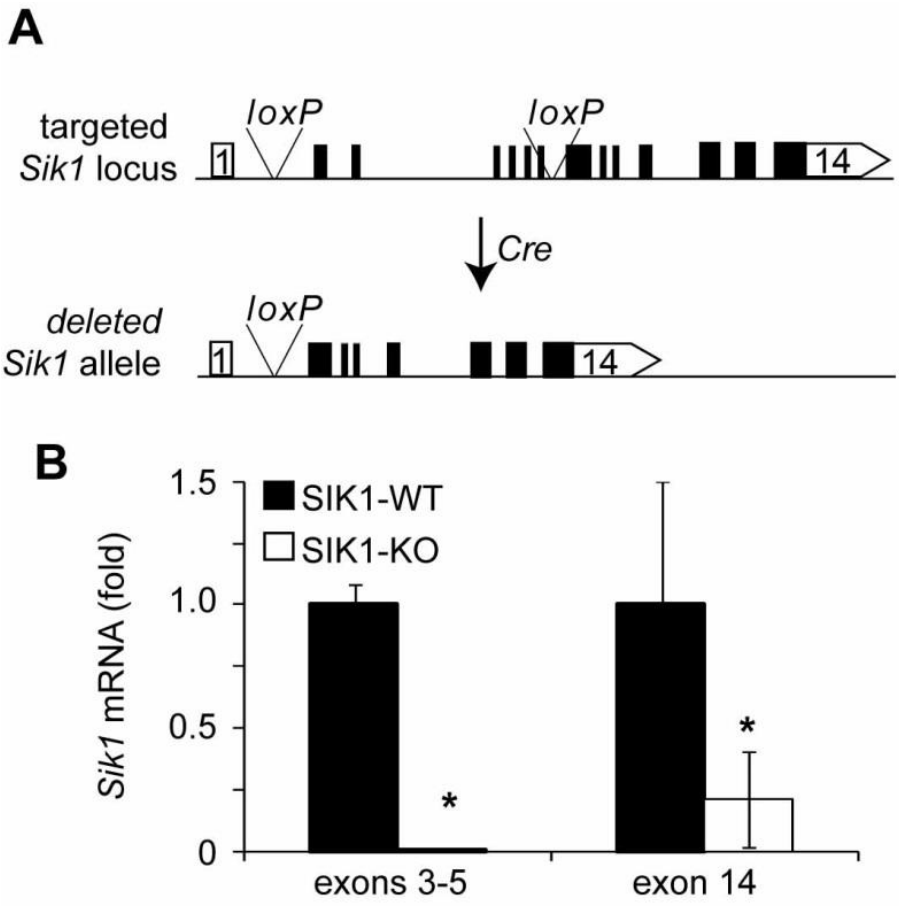

Figure 7. SIK1 conditional knockout mice. (A) Schematic of mouse *Sik1* locus with loxP sites shown. Cre-mediated recombination removes exons 2–7 (kinase domain). (B) qPCR of *Sik1* mRNA in the deleted region (exons 3–5) or intact genomic region (exon 14), normalized to *Rpl32*, fold of WT (mean ± stdev; n = 5 per genotype; *p < 0.05). Adapted from a figure published in Molecular Metabolism and used with journal permission: Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. Mol Metab 5: 34-46.

While my interests revolved around SIK1 action in skeletal muscle, it is important to remember that SIK1 is ubiquitously expressed (depending on the stress stimulus-

chapter 1), and we expected phenotypes in multiple tissues including liver and brain. For instance, we hypothesized that loss of SIK1 action in liver may cause hyperglycemia due to unrestrained gluconeogenesis (*de novo* glucose production from glycerol, lactate, and amino acids). SIK kinases are among thousands of CREB target genes (discussed in chapter 1, (11)) that are expressed in liver cells stimulated by the hormone glucagon (84). In addition to increased SIK expression, activation of CREB in the liver (S133 phosphorylation and recruitment of co-activators including CRTCs) causes transcription of rate-limiting gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (*Pepck*) and a catalytic subunit of glucose-6-phosphatase (*G6pase*) (85). Enhanced transcription of these factors leads to increased glucose production in liver cells (86, 87). SIK kinases have been observed to serve in a negative feedback loop to de-activate CRTCs (through phosphorylation on conserved 14-3-3 sites and nuclear export) (8, 88). This action ultimately serves to turn off CREB dependent transcription. In liver cells, inhibition of all SIK kinases (1,2 and 3) by HG-9-91-01 treatment was shown to correlate with reduced CRTC phosphorylation, increased gluconeogenic gene expression, and increased glucose production (89). Genetic deletion of *Sik2* alone or in combination with *Ampk* $\alpha 1/\alpha 2$ knockout (triple deletion) does not increase gluconeogenesis (89), but deletion of *Sik3* results in increased *Pepck* and *G6pase* mRNA expression in adult mouse liver despite enhanced AMPK expression (90). We were interested in determining whether *Sik1* deletion alone would activate the gluconeogenic program like SIK3 loss of function or whether compensation by other SIK and AMPK family members would occur. We used conditional SIK1 knockout mice for

this investigation. Intriguingly, our interests in SIK1 skeletal muscle action and in metabolism merged in an unexpected way.

4.2 Phenotypes of SIK1 deletion in unchallenged mice

4.2.1 SIK1-KO skeletal muscle development

We began phenotyping the SIK1 loss of function rodent model using global SIK1 knockout mice (*GDF9*-Cre). Mice were back-crossed with C57/Bl6 mice (Jackson) at least seven generations prior to testing. We obtained viable SIK1-KO male and female mice at slightly less than the expected Mendelian ratios (~10% reduction in homozygous SIK1-KO mice) and found that loss of *Sik1* did not affect gross longevity or health. Perhaps the most striking initial phenotype we observed in un-manipulated cohorts was reduced body size in SIK1-KO mice compared to wildtype littermates (23). This phenotype was also observed in a separate SIK1 knockout model (22) and is the subject of another study in our laboratory that will not be covered further in this text; however, it is an important factor we considered as we assessed metabolic phenotypes in these mice.

Since we hypothesized that deletion of *Sik1* would result in skeletal muscle defects, we immediately conducted extensive histological analyses of SIK1-KO skeletal muscle. Hematoxylin/Eosin staining of SIK1-KO muscle revealed nearly identical structural and organizational qualities of wildtype and SIK1-KO muscle (Fig. 8A) and Gömöri trichrome staining did not reveal accumulation of fibrous connective tissue in SIK1-KO muscles as we might expect with degenerative phenotypes (not shown). We did not find increased numbers of centrally-located nuclei (a hallmark of regenerated

muscle fibers) and we did not measure significant differences in gross muscle weights (Fig. 8B). Overall, there were no signs of muscle degeneration or impaired muscle development observed in SIK1-KO mice. Without data to support my hypothesis that SIK1-KO mice would have impaired muscle development, I began to investigate metabolic properties of our conditional SIK1 knockout mouse line for the reasons mentioned in the previous section.

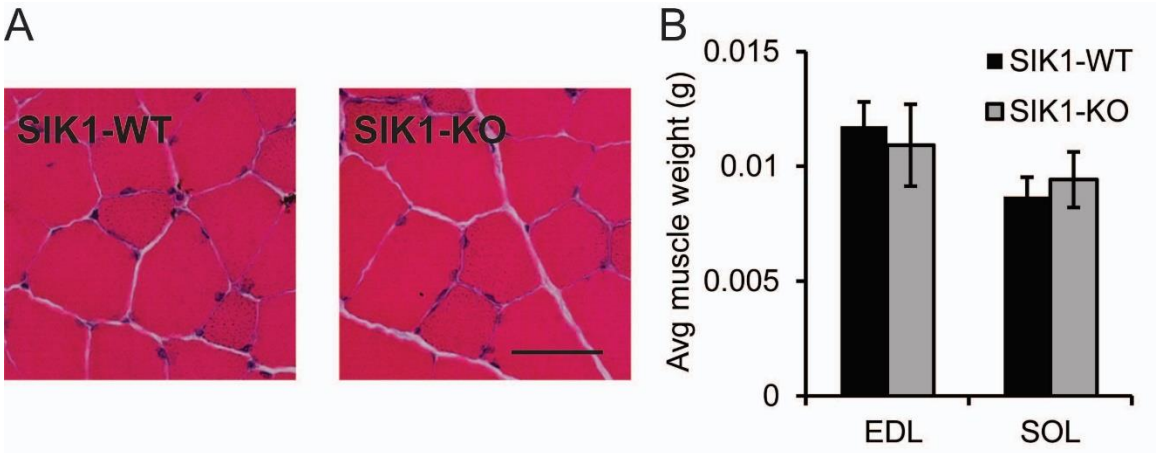

Figure 8. SIK1-KO skeletal muscle does not display impaired development. (A) H/E stained gastrocnemius muscle from adult wildtype (WT) and SIK1-KO mice, scale = 50μm. (B) Average muscle weights of extensor digitalis longus (EDL) and soleus muscles (SOL). Muscles were carefully disected under a microscope from tendon to tendon. Excess fat, debris, and buffer were removed before measurements were recorded, n = 6 per genotype.

4.2.2 SIK1-KO lean metabolism

When investigating metabolic characteristics of SIK1-KO mice, we found that lean adult males had modest reduction in *ad libitum* blood glucose levels but no change in fasting blood glucose levels (Fig 9A). Additionally, we found that SIK1-KO mice did not have altered glucose (Fig 9C) or insulin (Fig 9D) tolerance compared to wildtype littermates and did not display elevated gluconeogenic gene expression (Fig. 9B) (23).

This was surprising, as we expected loss of SIK1 to result in uninhibited glucose production in the liver and therefore elevated blood glucose levels. We observed no changes in lean mass or fat mass composition in global SIK1 knockout mice and observed no change in food intake or metabolic rate of SIK1-KO mice compared to wildtype controls. While conducting experiments to confirm *Sik1* deletion in SIK1-KO tissues, we noted that *Sik1* mRNA was expressed at such low levels in unchallenged mice that SIK1 proteins levels were difficult to even detect. In order to detect SIK1 in wildtype tissues, we had to immunoprecipitate the protein and load significant amounts of protein in electrophoretic gels. This observation made us hypothesize that phenotypes in SIK1-KO mice might only be evident under stressed conditions when endogenous SIK1 proteins is more abundant. We therefore considered relevant models of physiological stress in which SIK1 expression is elevated and the effects of impaired SIK1 action would likely be more apparent.

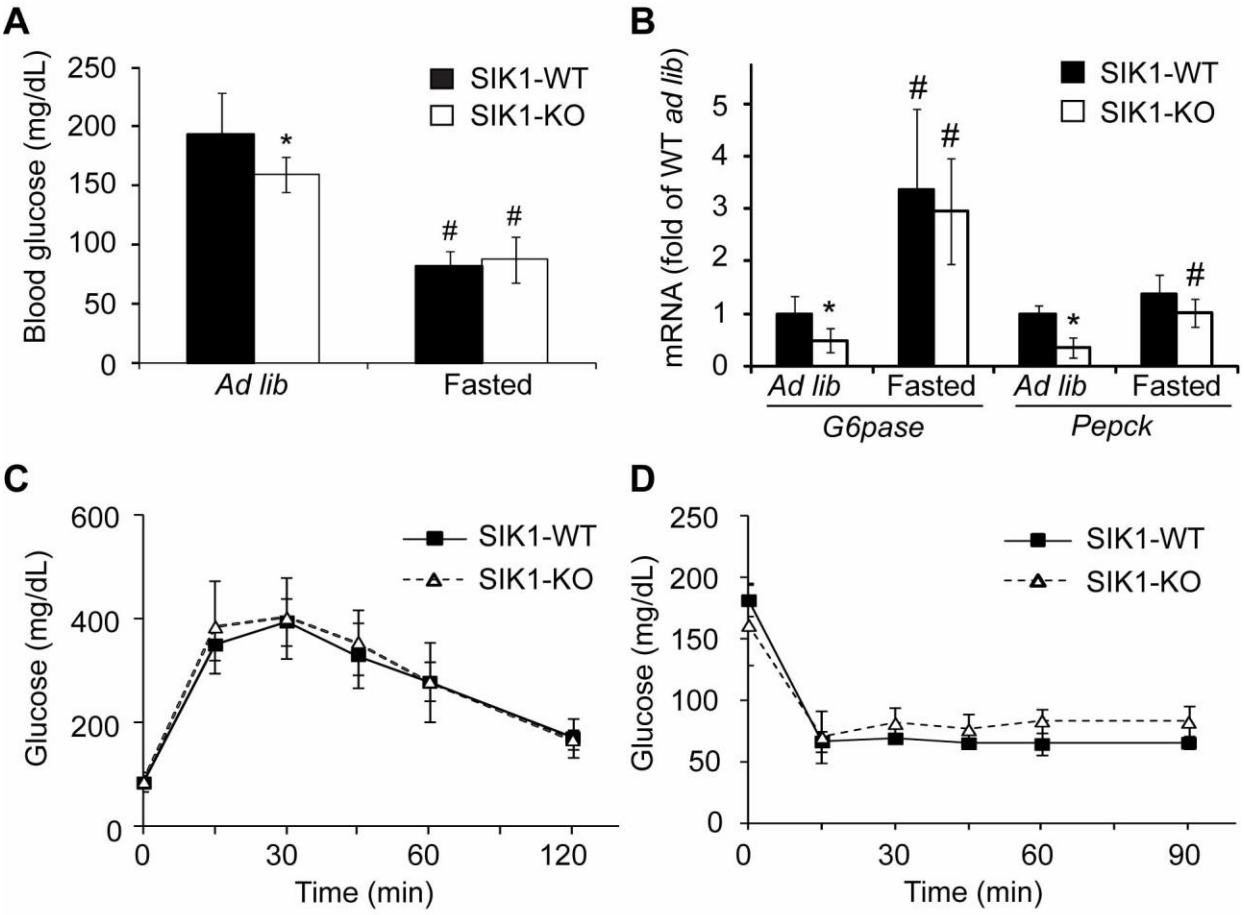

Figure 9. Lean SIK1-KO mice do not have increased gluconeogenesis. (A) *Ad libitum* and fasting blood glucose in global SIK1-KO mice (mean ± stdev; n = 10 per genotype; *p < 0.05 KO vs WT (ad lib); #p < 0.05 fasted vs ad lib within genotype). (B) Gluconeogenic gene expression in SIK1 knockout liver, ad libitum fed or after 16 h fasting, (mean ± stdev; n = 4–6 per genotype; *p < 0.05 KO vs WT (ad lib); #p < 0.05 fasted vs ad lib within genotype). (C) GTT of wild type and SIK1-KO mice (mean ± stdev; n = 5–6 per genotype). (D) ITT of SIK1-WT and SIK1-KO mice (mean ± stdev, n = 5–6 per genotype). Dr. Mark Nixon performed experiments in these panels. Randi Fitzgibbon and Dr. Nicholas Justice performed gene targetting to generate SIK1-KO mice. Adapted from a figure published in Molecular Metabolism and used with journal and co-author permission: Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. Mol Metab 5: 34-46.

As previously discussed, SIK1 is a stress inducible kinase. Part of the interest in SIKs results from the fact that the expression of these proteins is typically upregulated in response to stressful stimuli in order to re-establish homeostatic conditions. Since we were interested in SIK1 actions during myogenesis and in metabolism, we searched for SIK1-inducing stimuli in muscle. For our myogenesis study, we utilized our previous findings that demonstrated increased SIK1 expression in cardiotoxin injured skeletal muscle from wildtype mice (31). We conducted similar injury experiments in SIK1-KO mice to determine if loss of SIK1 impaired skeletal muscle regeneration.

4.3 Phenotypes of SIK1 deletion in challenged mice

4.3.1 SIK1-KO skeletal muscle myogenesis after injury

To investigate the effects of *Sik1* deletion during muscle injury and repair, we injected cardiotoxin (CTX) in one tibialis anterior muscle (TA) of adult male SIK1-KO and wildtype mice and collected injured (and un-injured contralateral TA) muscles at multiple time points (1, 5, and 14 days; 5 days shown in Fig 10). Surprisingly, we found that SIK1-KO mice recovered from necrosis just as efficiently as wildtype controls. In fact, we found that some SIK1-KO mice even had larger regenerated skeletal muscle fibers than controls. These data argue against impaired skeletal muscle myogenesis in SIK1-KO mice and support the notion that the cellular phenotypes we originally observed in cultured cells are more complicated in the *in vivo* model we created. It is likely that other skeletal muscle kinases compensated for the loss of SIK1 in the *in vivo* injury model but were unable to do so in cells removed from their niche. It is also possible that we missed a potential phenotype as a result of the injury model we chose.

Cardiotoxin causes muscle necrosis without damaging the underlying basal lamina structure. Future studies employing a more severe model of necrosis that would further disturb the natural niche, barium chloride injection for example, could possibly reveal a myogenic phenotype we missed.

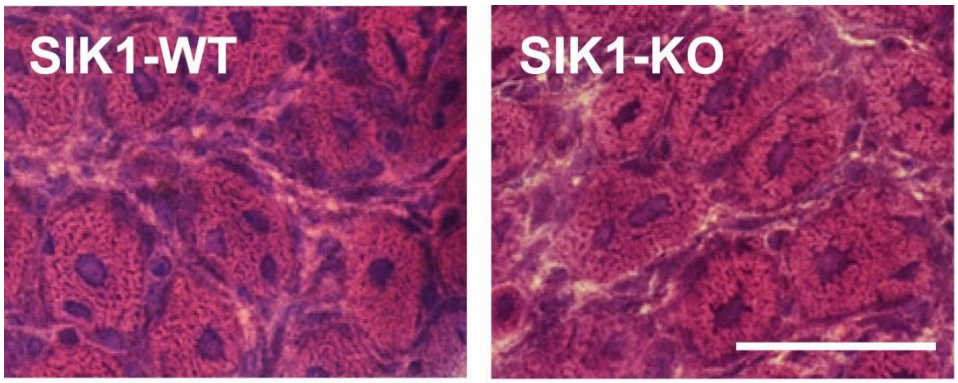

Figure 10. Skeletal muscle regeneration in SIK1-KO mice. H/E stains from adult male mice injected with CTX (25μL of 10μM stock) in tibialis anterior muscles. Muscles were allowed to regenerate for 5 days before tissue harvest, scale bar = 50μm. Representative of 5 mice per genotype.

In addition to toxin-induced injury, we tested SIK1-KO myogenic repair of muscle by crossing SIK1-KO mice with MDX mice (*Dmd*^{mdx}). MDX mice lack the structural protein Dystrophin and undergo continual rounds of muscle degeneration and regeneration beginning reliably at 4 weeks of age (91). This is a commonly used model in muscle regeneration studies because researchers know the mechanism and time line of muscle degeneration. We did not observe alterations in SIK1-KO degeneration or regeneration in this SIK1-KO:MDX model (not shown). From these studies, we concluded that SIK1 is dispensable for regeneration of mouse skeletal muscle.

In addition to injury related stimuli, I completed several experiments which employed exercise as a stimulus. I tested the functional characteristics of SIK1 null skeletal muscles and found reduced exercise capacity of SIK1-KO mice and reduced force generation in muscles excised from SIK1-KO males in collaboration with Dr. George Rodney's laboratory (Baylor College of Medicine). Additionally, I identified differences in MEF2 transcriptional activation after exercise in SIK1-KO mice crossed with Mef2LacZ reporter mice.

4.3.2 SIK1-KO in insulin resistance

One of the earliest published reports on SIK1 demonstrated upregulation of SIK1 expression in multiple tissues of obese *db/db* mice (5). These authors reported increased *Sik1* mRNA levels in both white and brown fat pads, liver, and skeletal muscle in their *db/db* cohorts. Since we were interested in identifying metabolically relevant stressors that increase SIK1 expression, and in the effects of SIK1 deletion on glucose metabolism, we adopted of model of obesity for phenotyping SIK1-KO mice. Unlike the previous study, we chose an alternate model of obesity that was not stimulated by genetically induced hyperphagy. We used a common diet-induced obesity (DIO) model which stimulates weight gain through increased calorie and fat intake. We found that this model of DIO induces *Sik1* expression in skeletal muscle (Fig11A). Briefly, mice were fed a diet high in fat (60% calories from fat) for 12-16 weeks to induce obesity and related metabolic disorder. Importantly, SIK1-KO mice became obese and gained a similar percentage of weight as wildtype controls despite starting with smaller body sizes. In agreement with Horike et al, we found that *Sik1* mRNA expression is dramatically up-regulated in skeletal muscles of obese wildtype mice compared to lean

controls (Fig 11). Additionally, we have since demonstrated that SIK1 protein is significantly increased in muscles of obese mice compared to lean controls (not shown). This latter finding is of particular importance given our prior characterization of SIK1 stability in skeletal muscle. With these findings, we had identified a clinically relevant model in which SIK1 protein is present at readily detectable levels and SIK1 kinase activity was presumably of consequence to metabolic balance.

We subsequently subjected obese control and SIK1-KO mice to metabolic testing. Interestingly, SIK1-KO mice displayed significantly enhanced glucose tolerance compared to obese wildtype controls in glucose tolerance tests (Fig 11B). This finding was contrary to our original hypothesis that loss of SIK1 would lead to un-controlled CREB dependent transcription of the gluconeogenic program and hyperglycemia. Although we found no difference in insulin tolerance in obese SIK1-KO mice, we did measure increased blood insulin levels (Fig 11C-D). This latter finding supports a report of increased insulin secretion from pancreatic β cells in a separate model (non-conditional) of *Sik1* deletion (22).

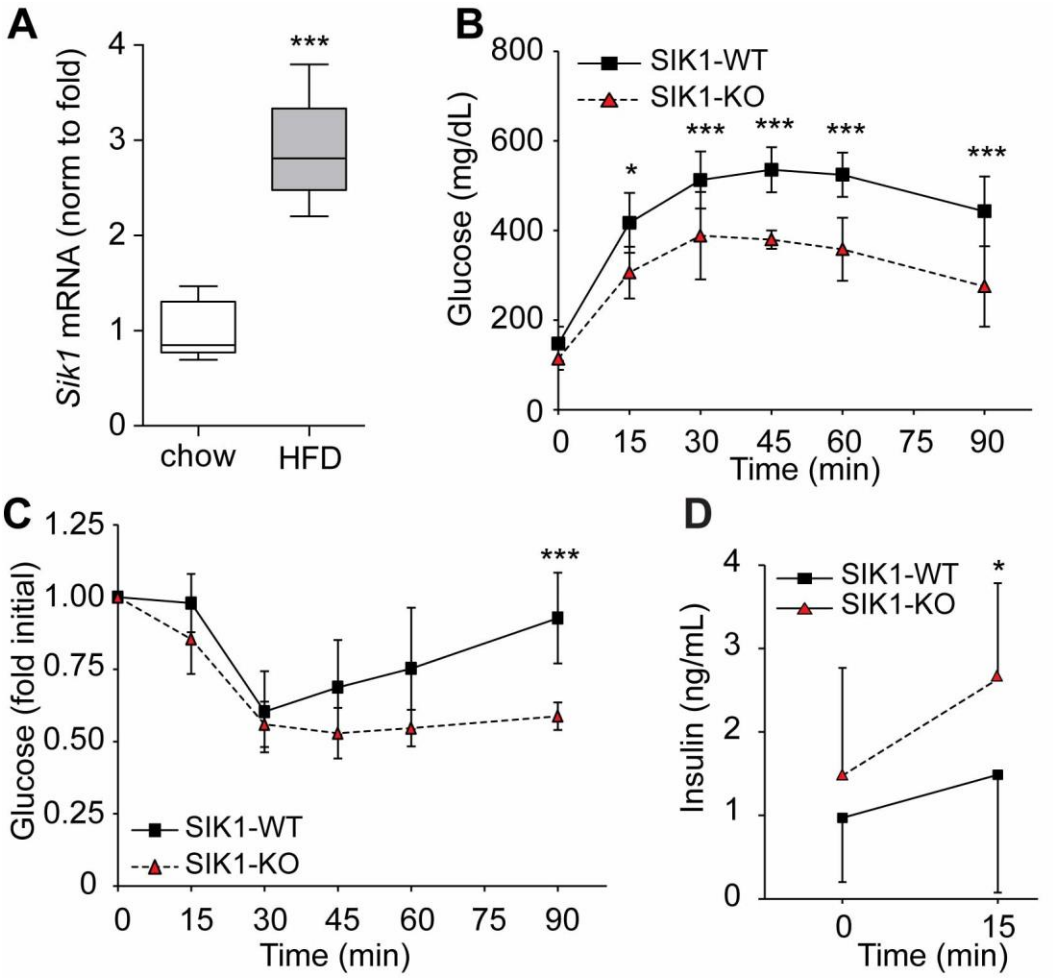

Figure 11. Improved glucose tolerance in obese SIK1-KO mice. (A) *Sik1* mRNA levels in quadriceps muscle in lean (chow fed) and obese (HFD fed) mice, normalized to *Gapdh* control. (B) GTT of male global SIK1-KO mice after 16–18 weeks of high fat diet feeding (mean ± stdev; n = 6–8 per genotype among 2 cohorts; 2-way repeated measures ANOVA $p < 0.0001$; Bonferroni post-tests: *$p < 0.05$; ***$p < 0.001$ vs WT at indicated time points). (C) ITT of male HFD SIK1-KO mice (mean ± stdev; n = 4–5 per genotype; 2-way repeated measures ANOVA $p = 0.06$; Bonferroni post-test: ***$p < 0.001$ vs WT at indicated time points). (D) Plasma insulin in obese SIK1-KO mice after overnight fast and 15 min after glucose injection (mean ± stdev; n = 11–12 per genotype among 2 cohorts; *$p < 0.05$). Dr. Mark Nixon performed experiments in these panels. Randi Fitzgibbon and Dr. Nicholas Justice performed gene targetting to generate SIK1-KO mice. Adapted from figures published in Molecular Metabolism and used with journal and co-author consent: Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. Mol Metab 5: 34-46.

Although increased plasma insulin levels could explain reduced blood glucose levels in SIK1-KO mice, we wondered whether there was an additional component of enhanced insulin sensitivity in SIK1-KO tissues. This is an important topic of investigation because advanced metabolic disorder and type 2 diabetes leads to hyperglycemia due to reduced insulin sensitivity in metabolic tissues despite abnormally high blood insulin concentrations (92). In order to address this possibility, we subjected a cohort of obese SIK1-KO and wildtype mice to hyperinsulinemic-euglycemic clamp testing. Through this experiment, we found that obese SIK1-KO mice have similar hepatic glucose output as controls (endogenous rate of glucose appearance, EndoR_a, Fig 12A) but require significantly more glucose infusion (glucose infusion rate, GIR) to sustain euglycemia in the presence of superphysiological blood insulin concentrations (Fig 12B). Importantly, the effects of increased β cell insulin secretion in obese SIK1-KO mice are eliminated once euglycemia is reached because blood insulin levels are so artificially high in both control and test animals that endogenous insulin levels become irrelevant. Additionally, we used a fluorinated glucose analog tracer to determine the biodistribution of glucose uptake in obese SIK1-KO and control mice. We measured insulin-stimulated glucose uptake in heart, white and brown fat, and skeletal muscle (quadriceps, gastrocnemius, and soleus). Interestingly, the only differences we observed in glucose consumption were in the large skeletal muscle groups (Fig 12C and not shown). Our findings confirm that obese SIK1-KO mice not only have increased blood insulin levels but also have enhanced insulin sensitivity with enhanced uptake into skeletal muscle. Drug targets with the ability to enhance insulin sensitivity in obese

states are of significant clinical interest as depressed insulin sensitivity is the crux of the type 2 diabetic disorder.

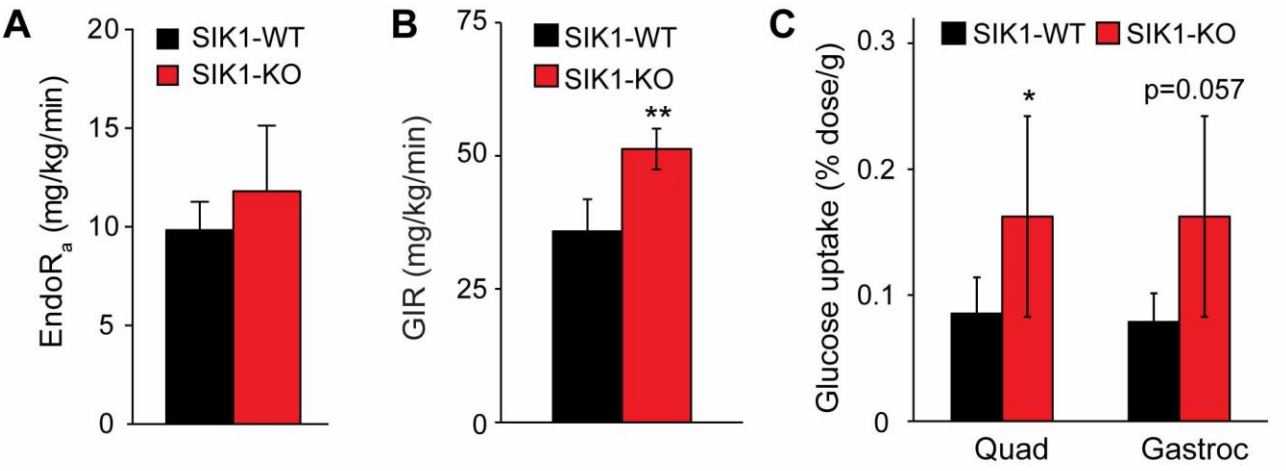

Figure 12. Improved insulin sensitivty in obese SIK1-KO mice. (A) Rate of endogenous glucose appearance (EndoRa) in fasted HFD male SIK1-WT and SIK1-KO mice 60 min after [3-3H]-glucose tracer infusion (mean ± stdev; n = 4–5 per genotype). (B) Glucose infusion rate (GIR) at the final time point of hyperinsulinemic euglycemic clamp in dynamic steady state (mean ± stdev; n = 4–5 per genotype; **p < 0.01). (C) Insulin-stimulated biodistribution of ^{18}F-fluorodeoxyglucose in obese male mice (mean ± stdev; n = 4–5 per genotype; *p < 0.05 vs WT). Adapted from a figure published in Molecular Metabolism and used with journal consent: Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. Mol Metab 5: 34-46.

4.3.3 SIK1 deletion in individual organs

There are many insulin-sensitive organs that remove glucose from the bloodstream in response to simulation by this hormone. Since obese SIK1-KO mice displayed enhanced insulin sensitivity, and therefore increased glucose uptake into peripheral tissues, we wondered which tissues consumed the excess glucose infused in our clamp experiment. In order to test this, we infused labeled glucose analog tracers into obese SIK1-KO and wildtype mice and measured the amount of tracer found in different tissue groups post insulin injection. Interestingly, the only tissues we found to

have significantly increased tracer concentrations in SIK1-KO mice were skeletal muscles (quadriceps and gastrocnemius) (Fig 12C). Glucose analog tracer concentrations were not statistically different in SIK1-KO heart or white or brown fat compared to controls. We previously knew that SIK1 was expressed in adult skeletal muscle and is highly regulated in this tissue but had not until this point realized that it played a role in skeletal muscle metabolism and glucose uptake. Importantly, increased glucose uptake specifically into skeletal muscle of obese SIK1-KO mice could be due to cell-autonomous effects of SIK1 in skeletal muscle or could be due to effects of *Sik1* deletion in other tissues that communicate with skeletal muscle to control glucose uptake in muscle. In order to decipher the true mechanism of increased skeletal muscle glucose uptake in the SIK1-KO model, we utilized the conditional nature of our mouse line and systematically deleted *Sik1* in tissues of interest before we subjected conditional knockout mice to Diet induced obesity and metabolic testing.

We deleted *Sik1* in liver (*Albumin*-Cre), brain (*Nestin*-Cre), white/brown fat (*Adiponectin*-Cre), and brown fat/skeletal muscle (*Myf5*-Cre). We originally used an *HSMA*-Cre (Human alpha-skeletal actin-Cre) mouse line to delete *Sik1* in skeletal muscle (and avoid deletion in other tissues) but unexpectedly found degenerative side effects in Cre control mice (wildtype SIK1 mice expressing HSMA-Cre) so utilized the *Myf5*-Cre mouse line to test the effects of SIK1 loss of function in muscle. It is not uncommon for tissue specific Cre mouse lines to lack exclusivity to a single tissue type. Since we were unable to use HSMA driven Cre, we were forced to use a driver that deletes targeted proteins in precursor cells for both skeletal muscle and brown fat (*Myf5*-Cre). We therefore used a delineative approach where we compared phenotypes

of fat-specific SIK1 knockout mice and muscle/fat SIK1 knockouts to determine the effects of SIK1 knockout in skeletal muscle specifically. For this reason, and to simplify terminology, *Myf5* driven SIK1-KO mice will further be termed “SIK1-MKO” (muscle knockout) mice. Importantly, this conditional model resulted in significant depletion of *Sik1* expression in mouse skeletal muscles (Fig 13A).

After metabolic testing of several conditional SIK1-KO lines, we found that only SIK1-MKO obese mice partially phenocopied the results originally observed in global SIK1-KO mice. Specifically, obese SIK1-MKO mice had improved glucose tolerance at later time points in glucose tolerance tests compared to Cre controls (Fig 13B) and higher glucose infusion rates under hyperinsulinemic-euglycemic clamp (Fig 13C). The results of SIK1-MKO glucose tolerance tests were not as dramatic as those observed in obese global SIK1-KO mice. This is likely due to loss of the hyper insulin secretion phenotype in SIK1-MKO mice (Fig 13D) because SIK1 action remains intact in β cells in this conditional model. Blood insulin levels were actually slightly depressed in obese SIK1-MKO mice compared to cre control mice. Additionally, it is important to note that SIK1-MKO mice lack the small body size phenotype observed in global SIK1-KO mice. This eliminates an important variable that could have played a significant role in our global SIK1-KO studies. Finally, infusion of glucose tracers in obese SIK1-MKO mice confirmed enhanced glucose uptake specifically in skeletal muscle lacking SIK1 (Fig 13E). These findings reveal cell autonomous action of SIK1 in skeletal muscle that normally inhibits glucose uptake into muscle in the context of obesity. Our findings position SIK1 as a potentially powerful therapeutic target to inhibit in patients suffering from T2DM. Inhibition of SIK1 in obese patients with metabolic disorder may serve to

enhance insulin sensitivity of skeletal muscle and reduced potentially life-threatening blood glucose levels.

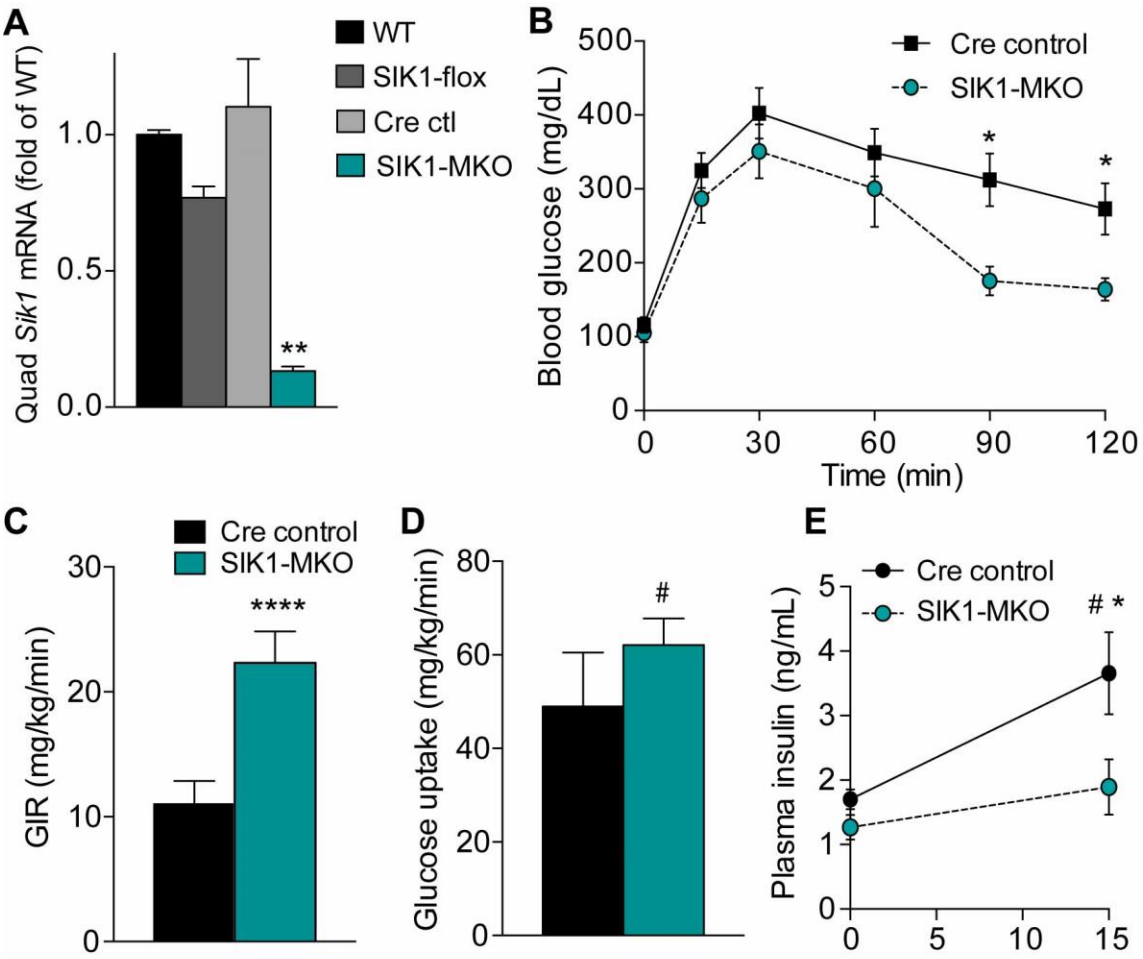

Figure 13. SIK1 regulates insulin sensitivity in a muscle cell autonomous fashion. (A) *Sik1* mRNA in quadriceps muscle of female wild type, floxed allele (no Cre expression), Cre control (Myf5Cre/+) and SIK1-MKO (mean ± stdev, n = 5 per genotype, ****, $p < 0.0001$) mice. (B) Glucose tolerance tests of male Cre control and SIK1-MKO fed 60% HFD for 12 weeks (mean ± SEM, n = 5–9 per genotype, $p < 0.05$ by 2-way repeated measures ANOVA, effect of genotype $p = 0.1$, *$p < 0.05$ at indicated time points by individual t-tests). (C) Glucose infusion rate (GIR) at final time point of dynamic steady state in hyperinsulinemic euglycemic clamps of male Cre control and SIK1-MKO mice fed HFD for 19 weeks (mean ± stdev, n = 5 per genotype, ****, $p < 0.0001$, *, $p < 0.05$ by t-test). (D) Skeletal muscle 2-deoxyglucose uptake rate during last 45 min of hyperinsulinemic euglycemic clamps (mean ± stdev, n = 5 per genotype, #, $p = 0.05$ by t-test). (E) Plasma insulin in male HFD fed SIK1-MKO mice, fasted (0) and 15 min after glucose injection IP (mean ± SEM, n = 4–5 per genotype, non-significant by 2-way RM ANOVA; *, $p < 0.05$ Cre:MKO by Sidak's multiple comparison test; #, $p < 0.05$ 0:15 min glucose in Cre control by t-test). Adapted from a figure published in Molecular Metabolism and used with journal consent: Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. Mol Metab 5: 34-46.

In order to provide pre-clinical data in support of SIK1 as a potential therapeutic target, we sought to determine the precise molecular mechanism by which SIK1 regulates blood glucose uptake into skeletal muscle. We first interrogated the potential effects of SIK1 on the well characterized insulin signaling pathway (discussed in chapter 1).

Chapter 5: Molecular mechanisms of SIK1-KO glucose uptake

5.1 Insulin signaling in SIK1 null skeletal muscle

The metabolic phenotypes we discovered in obese mice lacking SIK1 in skeletal muscle (enhanced insulin stimulated glucose uptake) position SIK1 as a potentially powerful therapeutic target for treating metabolic disorders like type 2 diabetes. Our research provides evidence that inhibition of SIK1 could be an effective strategy for avoiding insulin resistance associated with diet induced obesity. As a follow up to our physiological study of SIK1 loss of function in mice, we next sought to determine the molecular mechanism by which SIK1 regulates insulin sensitivity in the obese state. We conducted our research in skeletal muscle tissue since we had evidence that SIK1 regulation of glucose uptake occurs in a skeletal muscle cell autonomous fashion.

As discussed in chapter 1, intracellular insulin signaling is depressed in adipose and muscle cells of subjects suffering from advanced diabetes. This pathway and the alterations associated with insulin resistance are well characterized; therefore, the insulin signaling pathway served as a logical place to begin our search for molecular mechanism. We first investigated activation of the core insulin effector Protein kinase B (AKT). We used Ser473 and Thr308 phosphorylation-specific anti-AKT (pan) antibodies to gauge the level of AKT activation in muscle after acute insulin exposure (intraperitoneal injection). We found no significant enhancement of AKT phosphorylation at these critical sites in lean or obese SIK1-KO or SIK1-MKO mice compared to controls (Fig 14). Additionally, we did not observe differences in AKT phosphorylation between genotypes after prolonged insulin exposure post hyperinsulinemic-euglycemic clamp (not shown). Since we used antibodies that would recognize phosphorylation

modifications on all AKT isoforms, we wondered if we were missing alterations in phosphorylation of a specific AKT isoform. Since AKT2 is the dominant mediator of glucose import in skeletal muscle (93), we performed immunoprecipitation assays using an anti-AKT2 antibody to enrich this isoform in lysates. We then used the original phosphorylation-specific antibodies to measure AKT2 phosphorylation in western blots. Again, we did not observe differences in AKT activation in any of the samples and treatment conditions previously mentioned. In addition to probing AKT activation, we also explored activation (phosphorylation) of upstream insulin effectors: insulin receptor and insulin receptor substrate proteins (IRS). We did not detect differences in activation of these signaling molecules in any samples tested. This is not surprising since alterations in these upstream factors would likely result in similar alterations in AKT activation since it is a central node of the insulin signaling pathway.

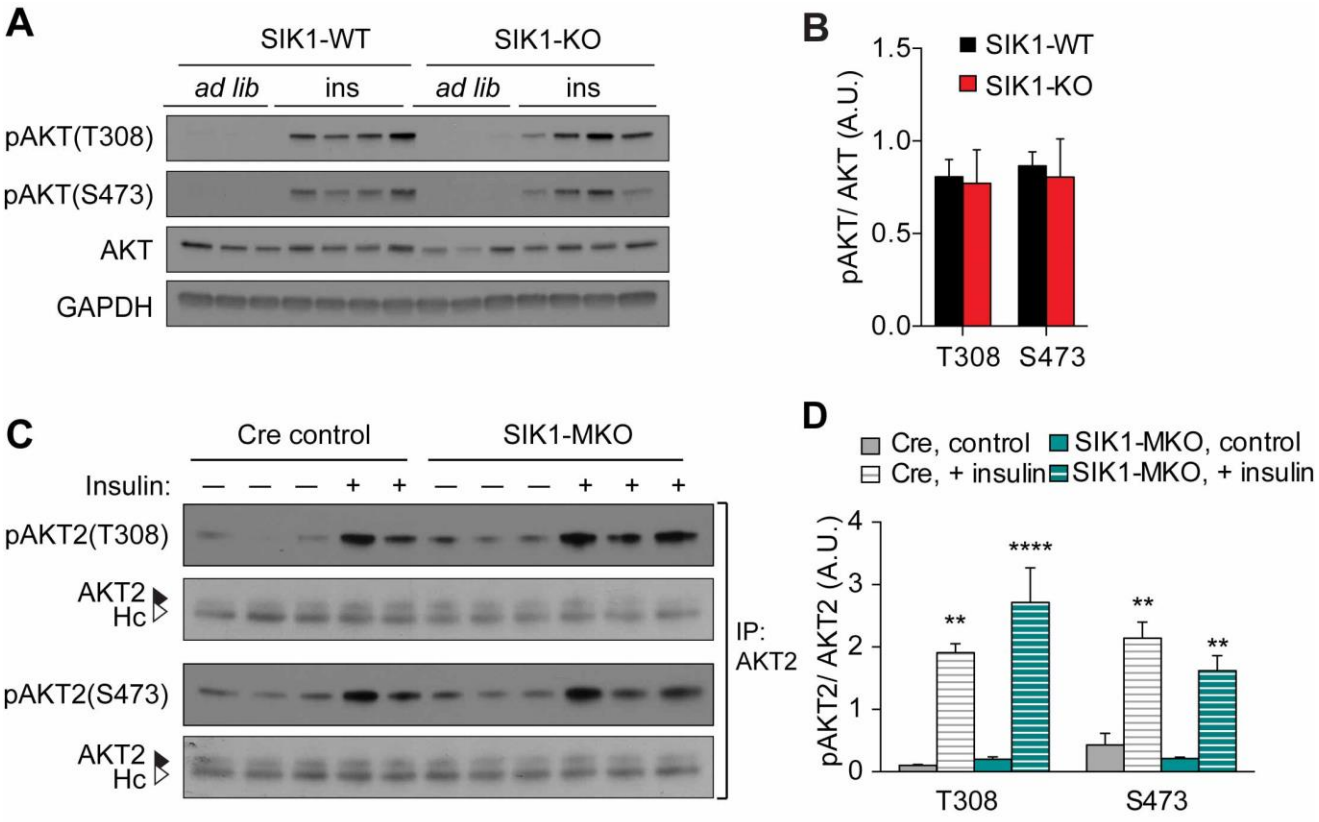

Figure 14. The canonical insulin signaling pathway is not enhanced in muscles from obese SIK1-KO or MKO mice. (A) Western blots of phosphorylated and total AKT in quadriceps muscle from male HFD-fed SIK1-WT and KO animals euthanized ad libitum or after 6 h fast and IP insulin (15 min). Each lane represents one animal (n = 4 per condition). (B) Densitometry of insulin treated mice from A. (C) Western blots of AKT2 immunoprecipitates (muscle) from male 12-week HFD-fed Cre control and MKO animals fasted for 6 h and/or administered insulin IP 15 min prior to euthanization. (D) Densitometry of panel C. Adapted from a figure published in Molecular Metabolism and used with journal permisson: Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. Mol Metab 5: 34-46.

Enhanced glucose uptake into skeletal muscle could occur independently of AKT if SIK1 were somehow affecting expression, recruitment, and/or incorporation of glucose transporters into the plasma membrane of skeletal muscle cells. We measured mRNA expression of GLUTs 1, 4, and 12 (Fig 15A) and did not detect differences between lean or obese mice of each genotype. Additionally, we did not observe changes in GLUT1 or GLUT4 protein levels in lean or obese SIK1-KO muscles compared to controls (not

shown). To determine if glucose transporters were more concentrated on membranes of SIK1-KO skeletal muscle cells than controls, we used anti-glucose transporter (GLUT 1 and 4) antibodies and secondary antibodies conjugated to immunofluorescent tags to image insulin treated muscle *ex vivo*. We did not observe differences between genotypes in GLUT4 (Fig 15B) or GLUT1 (not shown) localization to plasma membranes in skeletal muscles from obese mice. Since we were interested in identifying the molecular mechanism of sustained insulin sensitivity in SIK1 null muscles specifically during obesity, we considered mechanisms of glucose uptake regulation that are independent of the canonical insulin signaling pathway and GLUT trafficking.

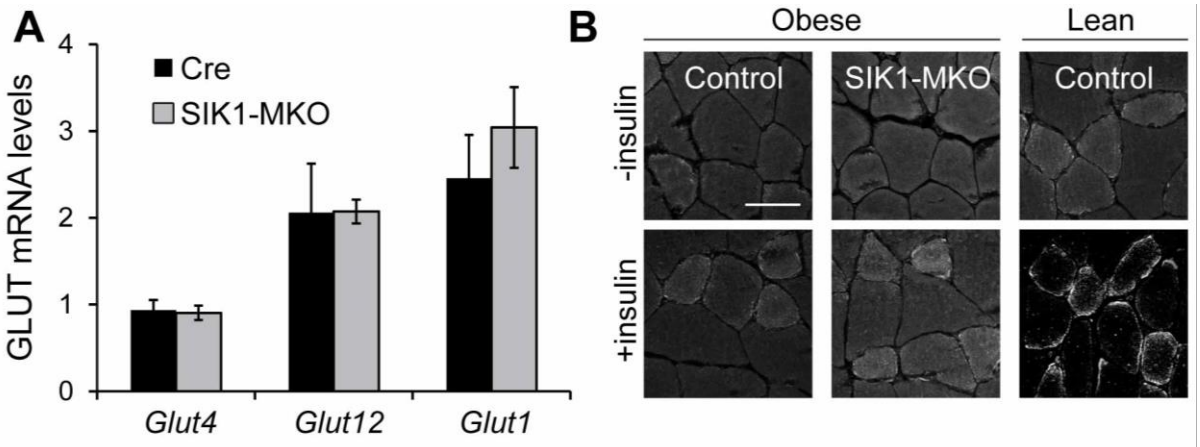

Figure 15. Glucose transporters in SIK1 null muscle. (A) mRNA expression levels of glucose transporters in quadriceps muscles from obese mice. GLUT expression levels were normalized to *Gapdh* expression for each mouse, n = 3 per genotype. No statistatical difference was found for any target between groups. (B) Immunofluorescence staining of quadriceps muscles from lean (insulin sensitive control) and obese mice +/- acute insulin treatment (6 hour fasting +/- 15 min intraperatoneal insulin injection 1U/kg). Scale = 50μm.

5.2 Fuel utilization in obese SIK1-KO skeletal muscle

Based on the Randle's cycle of metabolism (discussed in chapter 1), we postulated that glucose uptake in obese SIK1-KO mice could occur through changes in

the balance of fatty acid utilization versus glucose metabolism in skeletal muscle cells. Enhanced glucose uptake into skeletal muscle could be explained by reduced fatty acid utilization in this tissue. We did not observe overtly elevated lipid accumulation in quadriceps muscle from obese SIK1-KO mice compared to controls by Oil Red O staining (not shown). In collaboration with Olga Ilkayeva, Ph.D. (Director of the Metabolomics laboratory at Duke Molecular Physiology Institute), we examined the metabolite profiles of muscles from obese SIK1-KO and control mice. In SIK1-KO muscle, we did not measure changes in short chain acylcarnitines (Fig 16A) but did measure elevated medium and long chain acylcarnitine species (Fig 16B) along with elevated C11-Acyl CoA (Fig 16C), indicative of elevated intramyocellular fatty acid concentrations. We did not measure changes in DAG or ceramides (not shown). These data seem counterintuitive at first glance. Increased fatty acid utilization should presumably reduce glucose uptake in skeletal muscle and accumulation of fatty acid intermediates such as the acylcarnitines identified, have been linked to insulin resistance (71, 94-96). However, we have not yet tested the efficiency of mitochondrial import or the fate of these fatty acid intermediates to verify their full metabolism. Importantly, a report by Koves et. al. demonstrated that the fate of fatty acid intermediates (complete oxidation) is more important for determining insulin sensitivity than the actual concentrations of intracellular lipid species. This group showed that reduced β-oxidation serves to preserve insulin dependent glucose uptake in skeletal muscle (71). Therefore, future studies determining the precise intracellular fate of muscle metabolites will be important for determining whether alterations in cellular lipid metabolism are the cause of the phenotypes we observed in obese SIK1-KO mice.

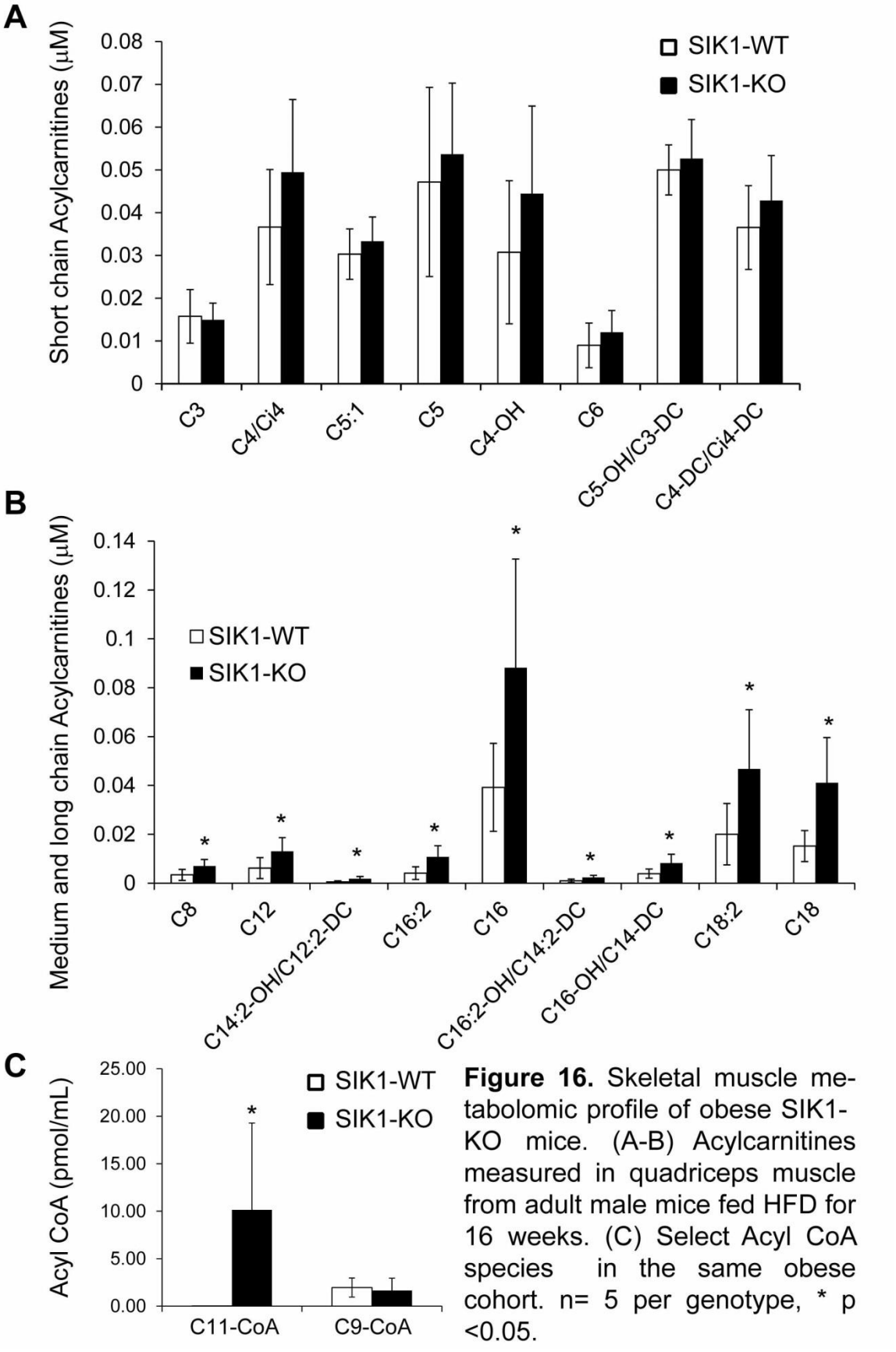

Figure 16. Skeletal muscle metabolomic profile of obese SIK1-KO mice. (A-B) Acylcarnitines measured in quadriceps muscle from adult male mice fed HFD for 16 weeks. (C) Select Acyl CoA species in the same obese cohort. n= 5 per genotype, * p <0.05.

In addition to our investigation of the intracellular lipid profile in muscles from obese SIK1-KO mice, we also measured key products along the pathway of glucose metabolism. We found no difference in glycogen concentrations in SIK1-KO muscles (Fig 17A) indicating that the excess glucose imported into SIK1-KO muscles is not stored and is indeed used for glycolysis. Additionally, we did not observe increased hexokinase activity in SIK1-KO muscle cells (not shown). We did not observe significant increases in pyruvate or lactate concentrations in SIK1-KO muscles, although there was a trend toward higher levels of these organic acids (Fig 17B-C). Finally, we did not detect changes in acetyl CoA concentrations (the product of both β-oxidation and pyruvate oxidation used to fuel the TCA cycle) (Fig 17D). This last finding is interesting given the evidence of greater glucose and fatty acid availability in SIK1-KO muscles. Either acetyl CoA is not being efficiently produced or is being consumed at a higher rate in SIK1-KO muscles compared to controls.

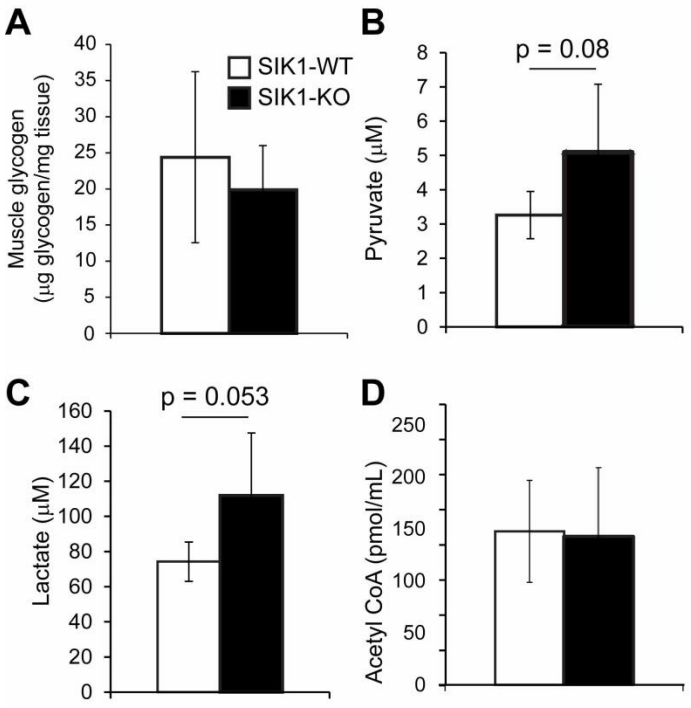

Figure 17. Glucose metabolites in skeletal muscle from obese SIK1-KO and control mice. (A) Glycogen concentrations in quadriceps muscles from *ad lib* fed adult male mice (16 weeks HFD). (B-C) organic acids and (D) total acetyl CoA concentrations in the same obese cohort. n= 5 per genotype.

Despite evidence of increased substrate availability (increased glucose uptake and intracellular lipid content), we surprisingly found reduced ATP concentrations in the skeletal muscles from HFD fed SIK1-KO mice (Fig 18). This observation demonstrates that although there are increased substrates for fuel production (fatty acids and glucose) within muscles, obese SIK1-KO mice are either unable to produce energy as efficiently as wild type controls or consume energy (ATP) at a significantly elevated rate. Since ATP serves as an allosteric regulator of glycolysis (97), we reason that ATP-depleted SIK1-KO muscle cells sense that they are in a state of starvation and serve as a glucose sink in an attempt to re-establish proper ATP concentrations.

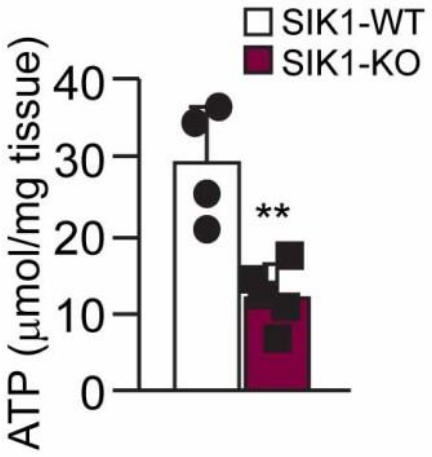

Figure 18. Reduced ATP concentrations in muscles from obese SIK1-KO mice. Male mice were fed high fat diet for 15-16 weeks and ATP levels were measured from quadriceps muscle with a luciferase assay (Sigma); *p = 0.03, n = 4 SIK1-WT and n = 5 SIK1-KO mice. A similar trend was also observed in a separate cohort of mice but data were not combined.

5.3 SIK1 regulates pyruvate dehydrogenase activity

Increased skeletal muscle glucose availability paired with no change in acetyl CoA concentrations and reduced ATP concentrations provoked us to consider potential flaws in SIK1-KO pyruvate oxidation. We therefore measured the activity of pyruvate dehydrogenase (PDH) which converts the fuel produced from glycolysis (pyruvate) into acetyl CoA which is further used to fuel the TCA cycle and ATP production. We found drastically reduced PDH activity in muscles from obese SIK1-KO mice compared to

wildtype controls (Fig 19A). The reduced enzymatic activity we observed likely results from reduced protein concentration since we also observed significantly reduced PDH1β protein levels in isolated mitochondria from the same SIK1-KO muscles (Fig 19B). Despite reduced protein levels, PDH1β mRNA levels are not different in muscles from obese SIK1-KO or MKO mice compared to controls. These data indicate possible post-translational regulation of PDH1β protein. Indeed, this protein has been demonstrated to be degraded by the proteasome (98, 99). It is surprising that we did not observe reduced acetyl CoA concentrations despite the significant reduction in PDH1β content and activity. The source of compensation is a subject of current investigation in our laboratory and has potential to explain the phenotypes we observed *in vivo*.

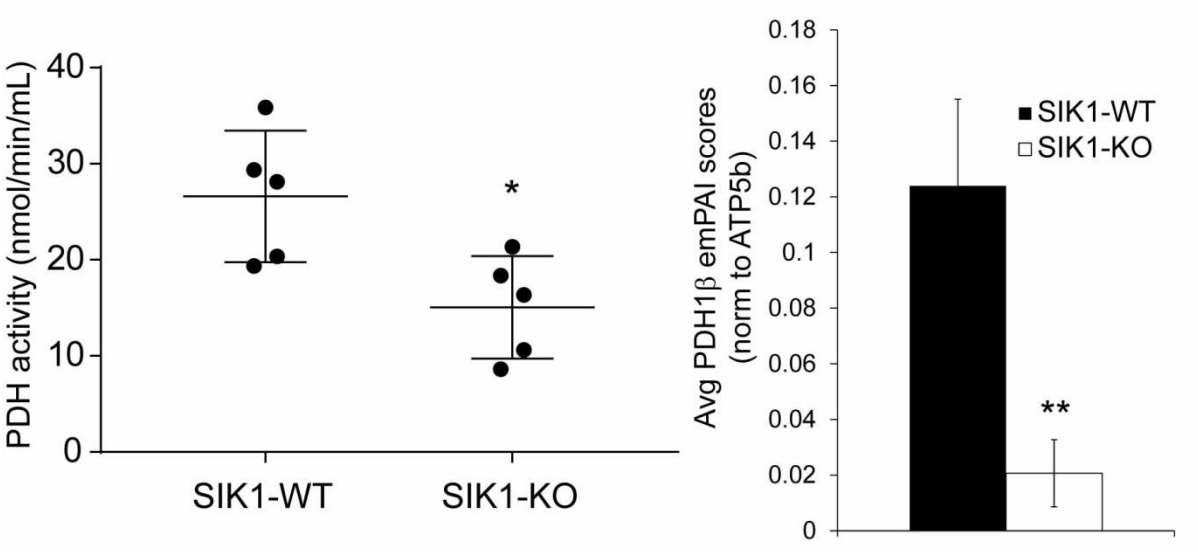

Figure 19. PDH1β is reduced in muscles from obese SIK1-KO mice. (A) PDH enzymatic activity in quadriceps muscles from HFD fed male mice (16 weeks diet), n = 5 per genotype, * p< 0.05. (B) Average emPAI scores (mass spectometry) for Pdh1β protein levels in lysates made from isolated mitochondria from obese mice, n = 5 per genotype, **p = 0.005.

One focus of our current research is to describe how possibly decreased glucose and/or fatty acid metabolism in SIK1-KO muscle could spur increased glucose uptake and preserve insulin sensitivity in muscle cells. One hypothesis is that SIK1-KO muscles take in excess glucose in an attempt to restore depleted ATP levels but are unable to detect the downstream PDH1β defect in glucose metabolic processing. Additionally, increased fatty acid oxidation in obese states has been proposed to overload mitochondria causing substantial stress and eventual mitochondrial dysfunction if substrate input continually exceeds mitochondrial capacity (71). Are SIK1-KO mitochondria stressed? Reduced intracellular ATP levels could reflect dysfunctional mitochondria or could serve to stimulate processes to enhance mitochondrial efficiency.

5.4 Mitochondrial stress in SIK1-KO muscle

Our findings reveal an interesting paradigm in which muscles with possibly defective glucose and/or fatty acid metabolism work to improve whole body glucose handling and reduce potentially life-threatening hyperglycemia. Reduced SIK1-KO intramuscular ATP concentrations and the contractility defects mentioned in chapter 4 are indicative of unhealthy muscle. We observed other signs of distress in SIK1 null skeletal muscle cells.

Since multiple SIK1 effectors modulate gene expression, we conducted a microarray experiment to identify alterations in SIK1-MKO and control expression profiles after DIO. The largest change in gene expression we observed was for the *Park2* gene which encodes the E3 ligase Parkin. Expression of *Park2* was upregulated several fold in quadriceps muscle from obese SIK1-MKO mice compared to Cre control

mice (Fig 20A). Up-regulation of this gene is of interest to our group because Parkin regulates mitochondrial fission and fusion dynamics and mitophagy which have been reported to change in the context of obesity (72, 100). Additionally, mitochondrial fission and fusion are dynamically regulated in many tissues to accommodate changes needed for efficient ATP production in response to stress (101, 102), so perhaps it is not surprising that Parkin would be upregulated in seemingly stressed skeletal muscle tissue like SIK1-KO muscles. In addition to increased mRNA levels of *Park2*, we also observed significantly higher Parkin protein levels in all samples lacking SIK1 (isolated muscle cells and lean and obese tissues from SIK1-KO mice) (Fig 20B). Additionally, we found elevated Parkin association with mitochondria isolated from obese SIK1-KO mice compared to controls (Fig 20C). Interestingly, we did not observe upregulation of the mitophagy pathway driven by Parkin mitochondrial recruitment and target ubiquitination (not shown). It is possible that Parkin has effects on mitochondrial proteins outside of the mitophagic process in this context or has effects in other cellular locations (e.g. at the plasma membrane to regulate fatty acid translocator CD36). Of particular interest, PDH1β was shown to associate with Parkin in a pull down experiment used to search for Parkin interacting proteins (111). It is quite possible that Parkin promotes post-translational degradation of PDH1β and SIK1 regulates PDH1β activity indirectly through Parkin. This is the subject of future studies in our laboratory and signifies research of potentially pivotal importance for our understanding of skeletal muscle glucose metabolism in the obese state.

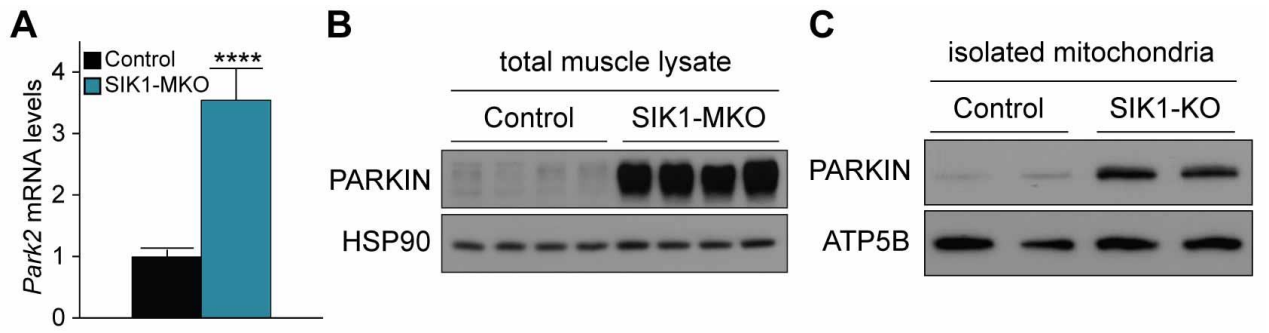

Figure 20. Elevated Parkin levels in SIK1-KO muscles. (A) Parkin mRNA and (B) protein levels in control and SIK1-null skeletal muscles from obese mice; n = 4 per genotype; ****p = $7.5x10^{-5}$. (C) Parkin protein levels specifically associated with mitochondria from control and SIK1-KO quadriceps muscle from obese mice.

5.5 Mitochondrial dynamics in SIK1-KO muscle

In the process of characterizing Parkin in SIK1-KO muscles, we noticed that mitochondrial organization appeared to differ in SIK1-KO and wildtype muscles. We observed hyperfusion of non-triad associated mitochondria in quadriceps and extensor digitalis longus (EDL) muscles of obese SIK1-KO mice by transmission electron microscopy (Fig 21). This phenotype seems counterintuitive since Parkin is up-regulated in these muscles and is thought to promote mitochondrial fission. This is further support, however, that the increased mitochondrial associated Parkin in SIK1-KO muscles may be playing a non-traditional role from those previously characterized. Additionally, hyperfusion of mitochondria in SIK1-KO muscles may be a compensatory result of ATP depletion. Indeed, mitochondrial hyperfusion has been shown to persist in stressed cells despite impaired activity of the electron transport chain (103).

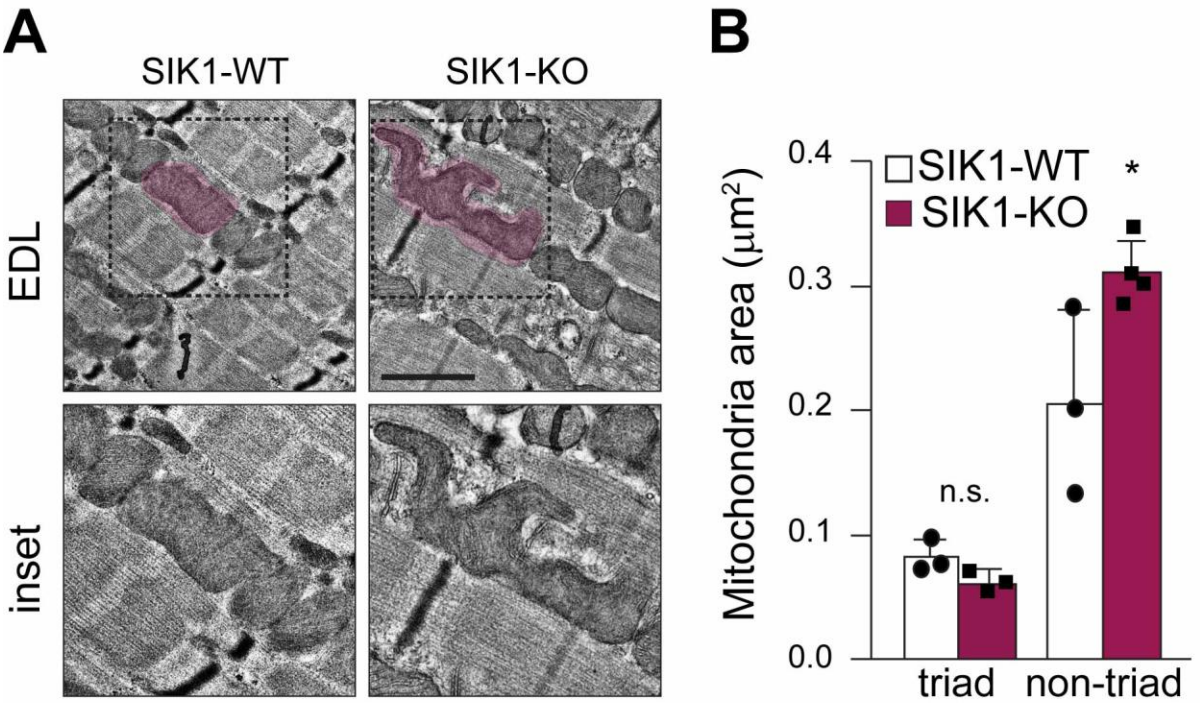

Figure 21. Hyperfusion of SIK1-KO mitochondria. (A) TEM images of quadriceps muscle from obese mice. Insets reflect the shaded areas in the top panel, scale = 1μm. (B) Average mitochondrial area of single mito organelles associated with muscle triads or non-triad associated mitochondria, $p<0.05$, n = 3-4 mice per genotype and 7 images per mouse.

Altogether, our current understanding of SIK1-KO mitochondrial organization and metabolic profile lead us to a model in which normal homeostatic balance of multiple intracellular processes is perturbed but leads to an unexpectedly beneficial outcome on whole body metabolism.

Chapter 6: Discussion and Future Directions

6.1 Current model and hypothesis

Our work has established SIK1 as a highly regulated protein kinase with a role in skeletal muscle glucose consumption and has revealed an interesting twist of possibly impaired glucose and fatty acid metabolism in the context of obesity. Loss of SIK1 in muscles of obese mice is associated with reduced ATP concentration, accumulation of fatty acid intermediates, depletion of PDH1β protein levels, and impaired pyruvate oxidation. On the surface, these metabolic deviations would seem to be conducive to the development of insulin resistance and hyperglycemia. Nevertheless, our SIK1-KO model demonstrates that these "defects" may ultimately function to enhance glucose uptake into skeletal muscle cells. Interestingly, our current model suggests that the systems sensing glucose availability, intracellular ATP concentrations and degree of β-oxidation in muscle lie upstream of SIK1 regulated metabolism. We hypothesize that the impaired glucose metabolism we observe at the level of PDH establishes a glucose sink that continually draws glucose into muscle cells and spits it back out as lactate (a glucose derivative a lot less toxic in plasma than glucose itself). Importantly, the relationship between PDH activity and the development of insulin resistance is currently a topic of debate in the glucose metabolism field (104-106). It is currently unclear as to whether SIK1 regulates PDH1β directly or if PDH1β inhibition is a secondary result of SIK1 deletion in muscle. A potentially powerful experiment for future research would be to test the phenotypic dependence of SIK1-KO mice on PDH1β inhibition using SIK1-KO x PDH kinase (2/4) double knockout mice which were reported to hyper-activate

PDH activity. Such a model would enable researchers to investigate whether reconstitution of PDH activity can rescue the mutant SIK1-KO phenotype (although it would be important to first determine whether activated PDH would still simply be degraded in SIK1-KO systems).

Another important possibility stemming from this research is the potential regulation of fatty acid metabolism by SIK1. We provide evidence of increased medium and long chain acylcarnitines within SIK1-KO muscle cells but have not tested the ability of such intermediates to enter mitochondria for full utilization. It is formally possible that despite fatty acid cellular influx, there is reduced fatty acid entry into mitochondria which could ultimately signal for increased glucose uptake (through ATP depletion over time). Indeed, the reduced ATP concentrations we observed in muscles from obese SIK1-KO mice are supportive of such a model. Future research on SIK1-KO carnitine carrier composition and function and efficiency of the TCA cycle and electron transport chain could prove valuable information for unlocking the molecular mechanism by which SIK1 regulates glucose uptake.

In addition to our hypotheses involving classic regulators of glucose metabolism, we also consider the potential role of Parkin and mitochondrial health and dynamics in the insulin sensitivity phenotype we've described. We hypothesize that *Park2* expression is elevated in SIK1-KO muscles because these appear to be stressed tissues. Parkin, like SIK1, is a stress induced molecule. It is quite possible that reduced oxidation and low ATP levels cause cells to operate as if they were starved despite abundant provisions. This sort of stress could be the driving force for increased Parkin expression and mitochondrial association. Indeed, cellular starvation has been shown to

enhance Parkin expression by several fold (107). Furthermore, we hypothesize that instead of participating in the most well characterized roles for Parkin (mitophagy and fission), this E3 ligase is playing an alternate role in our model. We are currently interested in investigating the ability of Parkin to ubiquitinate PDH1β to promote degradation of this enzyme in obese SIK1-KO mouse muscles. Indeed it has been reported that PDH1β interacts with Parkin (111). It remains unclear how *Park2* expression is increased in our knockout model. It is possible that SIK1 regulates modulator(s) of *Park2* expression or it is possible that *Park2* expression is upregulated secondarily to impaired mitochondrial ATP production, reduced PDH1β activity, and/or impaired mitochondrial fission through MFF1. These possibilities will be addressed in future work in our laboratory.

6.2 Significance of linking SIK1 to increased glucose metabolism

The first publications on SIK1 described this kinase as a gate keeper for homeostatic maintenance. In many instances, stressed cells upregulate SIK1 expression to restore homeostatic balance. Why would increased SIK1 expression in the obese state promote insulin resistance if the role of SIK1 is to maintain homeostasis? Since SIK1 is an evolutionarily conserved kinase that presumably played a role in metabolism of early man, I hypothesize that increased SIK1 expression was mainly up-regulated during times of starvation to promote fat break down and spare glucose for the brain. The stressful state of overnutrition was not likely a metabolic state ever encountered in early nomadic man. In modern society, food high in fat is readily available and consumed by many people on a regular basis. This leads to overnutrition and an imbalance in homeostasis that SIK1 cannot control. Mitochondrial overloading

due to severely increased demands for fatty acid oxidation likely leads to mitochondrial dysfunction and aberrant fat utilization in skeletal muscle that can eventually lead to insulin resistance.

6.3 SIK1 utility as a drug target to treat type 2 diabetes and unresolved questions

Our data support the use of SIK1 inhibitors in treating metabolic disorders associated with hyperglycemia. Importantly, deletion of the SIK1 kinase domain does not lead to developmental defects or obvious disorders. Pan SIK inhibitors such as HG-9-91-01 exist and have been used in animal studies; however, drugs that do not selectively inhibit SIK1 but rather inhibit all SIK family members would not be suitable therapeutics for treating hyperglycemia. SIK2 inhibition has been shown to subdue the insulin signaling pathway through IRS1 in fat tissue (5) and mice with global deletion of SIK2 display impaired glucose tolerance and insulin sensitivity (108). SIK3 inhibition (genetic deletion or inhibition with Pterosin B) results in uncontrolled gluconeogenic gene expression in hepatic cells (90, 109) and SIK3-KO mice display several detrimental phenotypes including impaired fat storage and defective cartilage formation (110).

Our research provides support for the use of SIK1 selective inhibitors to reduce blood glucose levels in obese patients. It is important to consider potential flaws in a SIK1 centered treatment strategy, however. For instance, SIK1 has been demonstrated as a positive mediator of the tumor suppressor p53 to avoid metastatic growth and low SIK1 expression has been associated with poor patient prognosis and increased epithelial-mesenchymal transition of tumor cells. For these reasons, it will be important

to invoke our conditional knock out model of SIK1 to delete this kinase in a time dependent manner, for prolonged intervals, and in additional models of cellular stress than presented here.

Our work utilizes a conditional knockout mouse model to inhibit SIK1 kinase activity prior to the induction of diet induced obesity. An important follow up experiment would be to use an inducible model of *Sik1* deletion that could be controlled in a time dependent manner to delete *Sik1* after obesity and insulin resistance are established. This type of model would be very clinically relevant for how SIK1 inhibitors would likely be used in human patients and would demonstrate whether the phenotypes we observed were solely due to proactive kinase inhibition. Additionally, we have not yet demonstrated whether SIK1 expression is sufficient to drive the development of insulin resistance. Our laboratory is currently developing models to test the hypothesis that over-expression of SIK1 is sufficient for the development of this pathology.

Despite our extensive characterization of SIK1 metabolic regulation, several questions remain to be answered at a later date. For instance, we are unsure about the precise SIK1 effector responsible for the glucose sink in SIK1-KO muscle. Rescue experiments expressing stable PDH1β or knock down of Parkin may serve to reveal the critical SIK1 effector. Additionally, it would be beneficial to determine PDH1β abundance and activity in SIK1-KO tissues other than skeletal muscle and much more detailed information about fatty acid utilization in SIK1-KO muscles could provide more mechanistic insight. We have evidence that Parkin is upregulated in multiple metabolically relevant tissues of SIK1-KO mice. It is possible that *Sik1* deletion effects metabolism in other tissues as well. For example, increased glucose uptake impairs

fatty acid release in adipose tissue. Indeed, global deletion of *Sik1* resulted in better glucose tolerance than deletion in skeletal muscle alone. Perhaps deletion of *Sik1* has additive effects in multiple tissues but was most apparent in skeletal muscle.

The work presented here provides strong evidence for continued investigation of SIK1 metabolic regulation and supports SIK1 as a potential clinical therapeutic.

Bibliography

1. Wang Z, Takemori H, Halder SK, Nonaka Y, Okamoto M. 1999. Cloning of a novel kinase (SIK) of the SNF1/AMPK family from high salt diet-treated rat adrenal. *FEBS Lett* 453: 135-9
2. Lin X, Takemori H, Katoh Y, Doi J, Horike N, Makino A, Nonaka Y, Okamoto M. 2001. Salt-inducible kinase is involved in the ACTH/cAMP-dependent protein kinase signaling in Y1 mouse adrenocortical tumor cells. *Mol Endocrinol* 15: 1264-76
3. Lin X, Takemori H, Doi J, Katoh Y, Okamoto M. 2000. SIK (Salt-inducible kinase): regulation of ACTH-mediated steroidogenic gene expression and nuclear/cytosol redistribution. *Endocr Res* 26: 995-1002
4. Doi J, Takemori H, Lin XZ, Horike N, Katoh Y, Okamoto M. 2002. Salt-inducible kinase represses cAMP-dependent protein kinase-mediated activation of human cholesterol side chain cleavage cytochrome P450 promoter through the CREB basic leucine zipper domain. *J Biol Chem* 277: 15629-37
5. Horike N, Takemori H, Katoh Y, Doi J, Min L, Asano T, Sun XJ, Yamamoto H, Kasayama S, Muraoka M, Nonaka Y, Okamoto M. 2003. Adipose-specific expression, phosphorylation of Ser794 in insulin receptor substrate-1, and activation in diabetic animals of salt-inducible kinase-2. *J Biol Chem* 278: 18440-7
6. Feldman JD, Vician L, Crispino M, Hoe W, Baudry M, Herschman HR. 2000. The salt-inducible kinase, SIK, is induced by depolarization in brain. *J Neurochem* 74: 2227-38
7. Giza CC, Prins ML, Hovda DA, Herschman HR, Feldman JD. 2002. Genes preferentially induced by depolarization after concussive brain injury: effects of age and injury severity. *J Neurotrauma* 19: 387-402
8. Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S, Yates JR, 3rd, Takemori H, Okamoto M, Montminy M. 2004. The CREB

coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. *Cell* 119: 61-74

9. Katoh Y, Takemori H, Lin XZ, Tamura M, Muraoka M, Satoh T, Tsuchiya Y, Min L, Doi J, Miyauchi A, Witters LA, Nakamura H, Okamoto M. 2006. Silencing the constitutive active transcription factor CREB by the LKB1-SIK signaling cascade. *FEBS J* 273: 2730-48
10. van der Linden AM, Nolan KM, Sengupta P. 2007. KIN-29 SIK regulates chemoreceptor gene expression via an MEF2 transcription factor and a class II HDAC. *EMBO J* 26: 358-70
11. Berdeaux R, Goebel N, Banaszynski L, Takemori H, Wandless T, Shelton GD, Montminy M. 2007. SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. *Nat Med* 13: 597-603
12. Dentin R, Liu Y, Koo SH, Hedrick S, Vargas T, Heredia J, Yates J, 3rd, Montminy M. 2007. Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. *Nature* 449: 366-9
13. Sjostrom M, Stenstrom K, Eneling K, Zwiller J, Katz AI, Takemori H, Bertorello AM. 2007. SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process. *Proc Natl Acad Sci U S A* 104: 16922-7
14. Kanyo R, Price DM, Chik CL, Ho AK. 2009. Salt-inducible kinase 1 in the rat pinealocyte: adrenergic regulation and role in arylalkylamine N-acetyltransferase gene transcription. *Endocrinology* 150: 4221-30
15. Cheng H, Liu P, Wang ZC, Zou L, Santiago S, Garbitt V, Gjoerup OV, Iglehart JD, Miron A, Richardson AL, Hahn WC, Zhao JJ. 2009. SIK1 couples LKB1 to p53-dependent anoikis and suppresses metastasis. *Sci Signal* 2: ra35
16. Stenstrom K, Takemori H, Bianchi G, Katz AI, Bertorello AM. 2009. Blocking the salt-inducible kinase 1 network prevents the increases in cell sodium transport caused by a hypertension-linked mutation in human alpha-adducin. *J Hypertens* 27: 2452-7

17. Romito A, Lonardo E, Roma G, Minchiotti G, Ballabio A, Cobellis G. 2010. Lack of sik1 in mouse embryonic stem cells impairs cardiomyogenesis by down-regulating the cyclin-dependent kinase inhibitor p57kip2. *PLoS One* 5: e9029

18. Wang B, Goode J, Best J, Meltzer J, Schilman PE, Chen J, Garza D, Thomas JB, Montminy M. 2008. The insulin-regulated CREB coactivator TORC promotes stress resistance in Drosophila. *Cell Metab* 7: 434-44

19. Choi S, Kim W, Chung J. 2011. Drosophila salt-inducible kinase (SIK) regulates starvation resistance through cAMP-response element-binding protein (CREB)-regulated transcription coactivator (CRTC). *J Biol Chem* 286: 2658-64

20. Eneling K, Brion L, Pinto V, Pinho MJ, Sznajder JI, Mochizuki N, Emoto K, Soares-da-Silva P, Bertorello AM. 2012. Salt-inducible kinase 1 regulates E-cadherin expression and intercellular junction stability. *FASEB J* 26: 3230-9

21. Stewart R, Akhmedov D, Robb C, Leiter C, Berdeaux R. 2013. Regulation of SIK1 abundance and stability is critical for myogenesis. *Proc Natl Acad Sci U S A* 110: 117-22

22. Kim MJ, Park SK, Lee JH, Jung CY, Sung DJ, Park JH, Yoon YS, Park J, Park KG, Song DK, Cho H, Kim ST, Koo SH. 2015. Salt-Inducible Kinase 1 Terminates cAMP Signaling by an Evolutionarily Conserved Negative-Feedback Loop in beta-Cells. *Diabetes* 64: 3189-202

23. Nixon M, Stewart-Fitzgibbon R, Fu J, Akhmedov D, Rajendran K, Mendoza-Rodriguez MG, Rivera-Molina YA, Gibson M, Berglund ED, Justice NJ, Berdeaux R. 2016. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. *Mol Metab* 5: 34-46

24. Sundberg TB, Liang Y, Wu H, Choi HG, Kim ND, Sim T, Johannessen L, Petrone A, Khor B, Graham DB, Latorre IJ, Phillips AJ, Schreiber SL, Perez J, Shamji AF, Gray NS, Xavier RJ. 2016. Development of Chemical Probes for Investigation of Salt-Inducible Kinase Function in Vivo. *ACS Chem Biol* 11: 2105-11

25. Sall J, Pettersson AM, Bjork C, Henriksson E, Wasserstrom S, Linder W, Zhou Y, Hansson O, Andersson DP, Ekelund M, Degerman E, Stenkula KG, Laurencikiene J, Goransson O. 2017. Salt-inducible kinase 2 and -3 are downregulated in adipose tissue from obese or insulin-resistant individuals: implications for insulin signalling and glucose uptake in human adipocytes. *Diabetologia* 60: 314-23

26. Mujahid N, Liang Y, Murakami R, Choi HG, Dobry AS, Wang J, Suita Y, Weng QY, Allouche J, Kemeny LV, Hermann AL, Roider EM, Gray NS, Fisher DE. 2017. A UV-Independent Topical Small-Molecule Approach for Melanin Production in Human Skin. *Cell Rep* 19: 2177-84

27. Heap RE, Hope AG, Pearson LA, Reyskens K, McElroy SP, Hastie CJ, Porter DW, Arthur JSC, Gray DW, Trost M. 2017. Identifying Inhibitors of Inflammation: A Novel High-Throughput MALDI-TOF Screening Assay for Salt-Inducible Kinases (SIKs). *SLAS Discov* 22: 1193-202

28. Kowanetz M, Lonn P, Vanlandewijck M, Kowanetz K, Heldin CH, Moustakas A. 2008. TGFbeta induces SIK to negatively regulate type I receptor kinase signaling. *J Cell Biol* 182: 655-62

29. Takemori H, Katoh Hashimoto Y, Nakae J, Olson EN, Okamoto M. 2009. Inactivation of HDAC5 by SIK1 in AICAR-treated C2C12 myoblasts. *Endocr J* 56: 121-30

30. Li S, Zhang C, Takemori H, Zhou Y, Xiong ZQ. 2009. TORC1 regulates activity-dependent CREB-target gene transcription and dendritic growth of developing cortical neurons. *J Neurosci* 29: 2334-43

31. Stewart R, Flechner L, Montminy M, Berdeaux R. 2011. CREB is activated by muscle injury and promotes muscle regeneration. *PLoS One* 6: e24714

32. Eneling K, Chen J, Welch LC, Takemori H, Sznajder JI, Bertorello AM. 2011. Salt-inducible kinase 1 is present in lung alveolar epithelial cells and regulates active sodium transport. *Biochem Biophys Res Commun* 409: 28-33

33. Dietrich JB, Takemori H, Grosch-Dirrig S, Bertorello A, Zwiller J. 2012. Cocaine induces the expression of MEF2C transcription factor in rat striatum through activation of SIK1 and phosphorylation of the histone deacetylase HDAC5. *Synapse* 66: 61-70

34. Liu Y, Poon V, Sanchez-Watts G, Watts AG, Takemori H, Aguilera G. 2012. Salt-inducible kinase is involved in the regulation of corticotropin-releasing hormone transcription in hypothalamic neurons in rats. *Endocrinology* 153: 223-33

35. Finsterwald C, Carrard A, Martin JL. 2013. Role of salt-inducible kinase 1 in the activation of MEF2-dependent transcription by BDNF. *PLoS One* 8: e54545

36. Jagannath A, Butler R, Godinho SI, Couch Y, Brown LA, Vasudevan SR, Flanagan KC, Anthony D, Churchill GC, Wood MJ, Steiner G, Ebeling M, Hossbach M, Wettstein JG, Duffield GE, Gatti S, Hankins MW, Foster RG, Peirson SN. 2013. The CRTC1-SIK1 pathway regulates entrainment of the circadian clock. *Cell* 154: 1100-11

37. Selvik LK, Rao S, Steigedal TS, Haltbakk I, Misund K, Bruland T, Prestvik WS, Laegreid A, Thommesen L. 2014. Salt-inducible kinase 1 (SIK1) is induced by gastrin and inhibits migration of gastric adenocarcinoma cells. *PLoS One* 9: e112485

38. Zhang Y, Takemori H, Wang C, Fu J, Xu M, Xiong L, Li N, Wen X. 2017. Role of salt inducible kinase 1 in high glucose-induced lipid accumulation in HepG2 cells and metformin intervention. *Life Sci* 173: 107-15

39. Jaitovich A, Bertorello AM. 2010. Intracellular sodium sensing: SIK1 network, hormone action and high blood pressure. *Biochim Biophys Acta* 1802: 1140-9

40. Yoon YS, Seo WY, Lee MW, Kim ST, Koo SH. 2009. Salt-inducible kinase regulates hepatic lipogenesis by controlling SREBP-1c phosphorylation. *J Biol Chem* 284: 10446-52

41. Yoshida H, Goedert M. 2012. Phosphorylation of microtubule-associated protein tau by AMPK-related kinases. *J Neurochem* 120: 165-76

42. Hu Z, Hu J, Shen WJ, Kraemer FB, Azhar S. 2015. A Novel Role of Salt-Inducible Kinase 1 (SIK1) in the Post-Translational Regulation of Scavenger Receptor Class B Type 1 Activity. *Biochemistry* 54: 6917-30

43. Qu C, He, Lu X, Dong L, Zhu Y, Zhao Q, Jiang X, Chang P, Wang L, Zhang Y, Bi L, He J, Peng Y, Su J, Zhang H, Huang H, Li Y, Zhou S, Qu Y, Zhao Y, Zhang Z. 2016. Salt-inducible Kinase (SIK1) regulates HCC progression and WNT/beta-catenin activation. *J Hepatol* 64: 1076-89

44. Ciciliot S, Schiaffino S. 2010. Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications. *Curr Pharm Des* 16: 906-14

45. Couteaux R, Mira JC, d'Albis A. 1988. Regeneration of muscles after cardiotoxin injury. I. Cytological aspects. *Biol Cell* 62: 171-82

46. Matsuura N, Cheng JS, Kalant N. 1975. Insulin control of hepatic glucose production. *Can J Biochem* 53: 28-36

47. Miller TB, Jr., Larner J. 1973. Mechanism of control of hepatic glycogenesis by insulin. *J Biol Chem* 248: 3483-8

48. Miller TB, Jr., Hazen R, Larner J. 1973. An absolute requirement for insulin in the control of hepatic glycogenesis by glucose. *Biochem Biophys Res Commun* 53: 466-74

49. Randle PJ. 1956. The assay of insulin in vitro by means of the glucose uptake of the isolated rat diaphragm. *J Endocrinol* 14: 82-6

50. Beloff-Chain A, Betto P, Catanzaro R, Chain EB, Longinotti L, Masi I, Pocchiari F. 1964. The metabolism of glucose 1-phosphate and glucose 6-phosphate and their influence on the metabolism of glucose in rat-diaphragm muscle. *Biochem J* 91: 620-4

51. Randle PJ, Garland PB, Hales CN, Newsholme EA. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1: 785-9

52. Uhles S, Moede T, Leibiger B, Berggren PO, Leibiger IB. 2003. Isoform-specific insulin receptor signaling involves different plasma membrane domains. *J Cell Biol* 163: 1327-37

53. Zhang B, Tavare JM, Ellis L, Roth RA. 1991. The regulatory role of known tyrosine autophosphorylation sites of the insulin receptor kinase domain. An assessment by replacement with neutral and negatively charged amino acids. *J Biol Chem* 266: 990-6

54. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* 7: 261-9

55. Scheid MP, Woodgett JR. 2003. Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett* 546: 108-12

56. Boucher J, Kleinridders A, Kahn CR. 2014. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb Perspect Biol* 6

57. Taniguchi CM, Emanuelli B, Kahn CR. 2006. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7: 85-96

58. Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, Lienhard GE. 2005. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem J* 391: 87-93

59. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE. 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278: 14599-602

60. Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE. 2002. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* 277: 22115-8

61. Richter EA, Hargreaves M. 2013. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93: 993-1017

62. Goodyear LJ, Hirshman MF, Valyou PM, Horton ES. 1992. Glucose transporter number, function, and subcellular distribution in rat skeletal muscle after exercise training. *Diabetes* 41: 1091-9

63. Klip A, Paquet MR. 1990. Glucose transport and glucose transporters in muscle and their metabolic regulation. *Diabetes Care* 13: 228-43

64. Kovacs P, Stumvoll M. 2005. Fatty acids and insulin resistance in muscle and liver. *Best Pract Res Clin Endocrinol Metab* 19: 625-35

65. Muoio DM, Newgard CB. 2008. Fatty acid oxidation and insulin action: when less is more. *Diabetes* 57: 1455-6

66. Li Y, Soos TJ, Li X, Wu J, Degennaro M, Sun X, Littman DR, Birnbaum MJ, Polakiewicz RD. 2004. Protein kinase C Theta inhibits insulin signaling by phosphorylating IRS1 at Ser(1101). *J Biol Chem* 279: 45304-7

67. Kim JK, Fillmore JJ, Sunshine MJ, Albrecht B, Higashimori T, Kim DW, Liu ZX, Soos TJ, Cline GW, O'Brien WR, Littman DR, Shulman GI. 2004. PKC-theta knockout mice are protected from fat-induced insulin resistance. *J Clin Invest* 114: 823-7

68. Haasch D, Berg C, Clampit JE, Pederson T, Frost L, Kroeger P, Rondinone CM. 2006. PKCtheta is a key player in the development of insulin resistance. *Biochem Biophys Res Commun* 343: 361-8

69. Hue L, Taegtmeyer H. 2009. The Randle cycle revisited: a new head for an old hat. *Am J Physiol Endocrinol Metab* 297: E578-91

70. Bouzakri K, Austin R, Rune A, Lassman ME, Garcia-Roves PM, Berger JP, Krook A, Chibalin AV, Zhang BB, Zierath JR. 2008. Malonyl CoenzymeA decarboxylase regulates lipid and glucose metabolism in human skeletal muscle. *Diabetes* 57: 1508-16

71. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM. 2008. Mitochondrial overload and

incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 7: 45-56

72. Jheng HF, Tsai PJ, Guo SM, Kuo LH, Chang CS, Su IJ, Chang CR, Tsai YS. 2012. Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle. *Mol Cell Biol* 32: 309-19
73. Montgomery MK, Turner N. 2015. Mitochondrial dysfunction and insulin resistance: an update. *Endocr Connect* 4: R1-R15
74. Drew BG, Ribas V, Le JA, Henstridge DC, Phun J, Zhou Z, Soleymani T, Daraei P, Sitz D, Vergnes L, Wanagat J, Reue K, Febbraio MA, Hevener AL. 2014. HSP72 is a mitochondrial stress sensor critical for Parkin action, oxidative metabolism, and insulin sensitivity in skeletal muscle. *Diabetes* 63: 1488-505
75. Sims NR, Anderson MF. 2008. Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. *Nat Protoc* 3: 1228-39
76. Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G. 2010. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J Clin Invest* 120: 11-9
77. Horike N, Takemori H, Katoh Y, Doi J, Okamoto M. 2002. Roles of several domains identified in the primary structure of salt-inducible kinase (SIK). *Endocr Res* 28: 291-4
78. Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M. 2005. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* 437: 1109-11
79. Potthoff MJ, Arnold MA, McAnally J, Richardson JA, Bassel-Duby R, Olson EN. 2007. Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. *Mol Cell Biol* 27: 8143-51
80. Meadows E, Cho JH, Flynn JM, Klein WH. 2008. Myogenin regulates a distinct genetic program in adult muscle stem cells. *Dev Biol* 322: 406-14

81. Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75: 1351-9

82. Lakso M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci U S A* 89: 6232-6

83. Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26: 99-109

84. Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M. 2001. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 413: 179-83

85. Dentin R, Hedrick S, Xie J, Yates J, 3rd, Montminy M. 2008. Hepatic glucose sensing via the CREB coactivator CRTC2. *Science* 319: 1402-5

86. Trinh KY, O'Doherty RM, Anderson P, Lange AJ, Newgard CB. 1998. Perturbation of fuel homeostasis caused by overexpression of the glucose-6-phosphatase catalytic subunit in liver of normal rats. *J Biol Chem* 273: 31615-20

87. Sun Y, Liu S, Ferguson S, Wang L, Klepcyk P, Yun JS, Friedman JE. 2002. Phosphoenolpyruvate carboxykinase overexpression selectively attenuates insulin signaling and hepatic insulin sensitivity in transgenic mice. *J Biol Chem* 277: 23301-7

88. Katoh Y, Takemori H, Min L, Muraoka M, Doi J, Horike N, Okamoto M. 2004. Salt-inducible kinase-1 represses cAMP response element-binding protein activity both in the nucleus and in the cytoplasm. *Eur J Biochem* 271: 4307-19

89. Patel K, Foretz M, Marion A, Campbell DG, Gourlay R, Boudaba N, Tournier E, Titchenell P, Peggie M, Deak M, Wan M, Kaestner KH, Goransson O, Viollet B, Gray NS, Birnbaum MJ, Sutherland C, Sakamoto K. 2014. The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver. *Nat Commun* 5: 4535

90. Uebi T, Itoh Y, Hatano O, Kumagai A, Sanosaka M, Sasaki T, Sasagawa S, Doi J, Tatsumi K, Mitamura K, Morii E, Aozasa K, Kawamura T, Okumura M, Nakae J, Takikawa H, Fukusato T, Koura M, Nish M, Hamsten A, Silveira A, Bertorello AM, Kitagawa K, Nagaoka Y, Kawahara H, Tomonaga T, Naka T, Ikegawa S, Tsumaki N, Matsuda J, Takemori H. 2012. Involvement of SIK3 in glucose and lipid homeostasis in mice. *PLoS One* 7: e37803

91. Dangain J, Vrbova G. 1984. Muscle development in mdx mutant mice. *Muscle Nerve* 7: 700-4

92. Wilcox G. 2005. Insulin and insulin resistance. *Clin Biochem Rev* 26: 19-39

93. Cleasby ME, Reinten TA, Cooney GJ, James DE, Kraegen EW. 2007. Functional studies of Akt isoform specificity in skeletal muscle in vivo; maintained insulin sensitivity despite reduced insulin receptor substrate-1 expression. *Mol Endocrinol* 21: 215-28

94. Muoio DM, Koves TR. 2007. Lipid-induced metabolic dysfunction in skeletal muscle. *Novartis Found Symp* 286: 24-38; discussion -46, 162-3, 96-203

95. Muoio DM, Neufer PD. 2012. Lipid-induced mitochondrial stress and insulin action in muscle. *Cell Metab* 15: 595-605

96. Schooneman MG, Vaz FM, Houten SM, Soeters MR. 2013. Acylcarnitines: reflecting or inflicting insulin resistance? *Diabetes* 62: 1-8

97. Barford D, Hu SH, Johnson LN. 1991. Structural mechanism for glycogen phosphorylase control by phosphorylation and AMP. *J Mol Biol* 218: 233-60

98. Kanda A, Noda K, Ishida S. 2015. ATP6AP2/(pro)renin receptor contributes to glucose metabolism via stabilizing the pyruvate dehydrogenase E1 beta subunit. *J Biol Chem* 290: 9690-700

99. Han Z, Zhong L, Srivastava A, Stacpoole PW. 2008. Pyruvate dehydrogenase complex deficiency caused by ubiquitination and proteasome-mediated degradation of the E1 subunit. *J Biol Chem* 283: 237-43

100. Gonzalez-Franquesa A, Patti ME. 2017. Insulin Resistance and Mitochondrial Dysfunction. *Adv Exp Med Biol* 982: 465-520

101. Herlan M, Bornhovd C, Hell K, Neupert W, Reichert AS. 2004. Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor. *J Cell Biol* 165: 167-73

102. Westermann B. 2012. Bioenergetic role of mitochondrial fusion and fission. *Biochim Biophys Acta* 1817: 1833-8

103. Legros F, Lombes A, Frachon P, Rojo M. 2002. Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. *Mol Biol Cell* 13: 4343-54

104. Petersen MC, Rahimi Y, Camporez JP, Pesta D, Perry RJ, Jurczak MJ, Cline GW, Shulman GI. 2015. Reply to Constantin-Teodosiu et al.: mice with genetic PDH activation are not protected from high-fat diet-induced muscle insulin resistance. *Proc Natl Acad Sci U S A* 112: E825

105. Constantin-Teodosiu D, Stephens FB, Greenhaff PL. 2015. Perpetual muscle PDH activation in PDH kinase knockout mice protects against high-fat feeding-induced muscle insulin resistance. *Proc Natl Acad Sci U S A* 112: E824

106. Rahimi Y, Camporez JP, Petersen MC, Pesta D, Perry RJ, Jurczak MJ, Cline GW, Shulman GI. 2014. Genetic activation of pyruvate dehydrogenase alters oxidative substrate selection to induce skeletal muscle insulin resistance. *Proc Natl Acad Sci U S A* 111: 16508-13

107. Klinkenberg M, Gispert S, Dominguez-Bautista JA, Braun I, Auburger G, Jendrach M. 2012. Restriction of trophic factors and nutrients induces PARKIN expression. *Neurogenetics* 13: 9-21

108. Park J, Yoon YS, Han HS, Kim YH, Ogawa Y, Park KG, Lee CH, Kim ST, Koo SH. 2014. SIK2 is critical in the regulation of lipid homeostasis and adipogenesis in vivo. *Diabetes* 63: 3659-73

109. Itoh Y, Sanosaka M, Fuchino H, Yahara Y, Kumagai A, Takemoto D, Kagawa M, Doi J, Ohta M, Tsumaki N, Kawahara N, Takemori H. 2015. Salt-inducible Kinase 3 Signaling Is Important for the Gluconeogenic Programs in Mouse Hepatocytes. *J Biol Chem* 290: 17879-93

110. Sasagawa S, Takemori H, Uebi T, Ikegami D, Hiramatsu K, Ikegawa S, Yoshikawa H, Tsumaki N. 2012. SIK3 is essential for chondrocyte hypertrophy during skeletal development in mice. *Development* 139: 1153-63

111. Pu, C. H. 2009. Quantitative mass spectrometry to discover interactors of parkin E3 ubiq uitin ligase, a protein implicated in early-onset Parkinson's disease (T). Electronic These s and Dissertations (ETDs) 2008+. University of British Columbia. Retrieved November 1 9, 2017, from https://open.library.ubc.ca/cIRcle/collections/24/items/1.0068961

Vita

Randi Nicole Fitzgibbon, the daughter of Judith Ann Stewart, was born in Great Falls, Montana in December 1985. She attended Winston Churchill High School in San Antonio, Texas and subsequently completed a Bachelor's in Science degree at Trinity University with a major in neuroscience. Randi worked as a research technician and lab manager in Dr. Rebecca Berdeaux's laboratory at UT Health Science Center at Houston (department of Integrative Biology and Pharmacology) for a little over 3 years before matriculating to the Graduate School of Biomedical Sciences in August 2012.