METABOLIC CHARACTERIZATION OF MICE LACKING CARBONIC ANHYDRASE III

Sarah Whitney Renner

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Genetics and Molecular Biology Curriculum

Chapel Hill
2017

Approved by:

Jay Brenman

Karen Mohlke

Daniel Pomp

Rosalind Coleman

Scott Bultman

ABSTRACT

Sarah Whitney Renner: Metabolic characterization of mice lacking carbonic anhydrase III (Car3)
(Under the direction of Jay Brenman)

Carbonic anhydrases (CAs) are a family of enzymes that catalyze the reversible condensation of water and carbon dioxide to bicarbonate and a proton. While CA III shares homology with α-CA family members, it has only 0.16% of the catalytic activity compared to other CAs. Despite the abundance of CA III in many metabolic tissues that are able to store lipids, a physiological role for CA III is currently unclear. Since CA III is nutritionally regulated, we utilized mouse genetics to evaluate the possible role of CA III in metabolism. Even though we found no obvious differences in metabolic phenotypes of mice deficient in CA III, we eliminated a potential function: CA III is not required for *de novo* lipogenesis. The role of CA III continues to be elusive, however it remains to be seen what phenotypes CA III deficient mice have under abnormal laboratory conditions.

ACKNOWLEDGMENTS

I would like to acknowledge Jay Brenman for the training opportunities, demanding my best scientific work, and teaching me many life lessons. I would also like to thank my current and former lab members (Lauren Walker, Cassie Meeker, Andrew Alin, and John Forsberg) for not only assisting in the collection of data but also their friendship. I would particularly like to thank John Forsberg for his guidance and thoughtful scientific and political discussions.

I wish to thank all of my thesis committee members: Karen Mohlke, Daniel Pomp, Rosalind Coleman, Scott Bultman, and Jonny Sexton, not only for teaching me quantitative genetics and nutritional metabolism in my early years but also for supporting me through the ups and downs of graduate school.

Graduate school has been incredibly challenging; the support and cheerleading from the graduate students, both in my cohort and in the Science and Business club, have been critical to my success. Particularly, I would like to thank Lydia Smith. Whether she was teaching me how to use Illustrator or Prism, spotting me on the bench press at the gym, or just being my sympathetic friend, I deeply value her support.

I would also like to thank my wonderful friends and family who have been so patient and understanding. This process can be isolating, so a special thank you to all of my friends who encouraged me from afar through phone calls, texts, and letters. A special thanks to Michelle Phillips, earlier in graduate school she helped me have faith in myself and has continued to rally my spirits. As for my family, my parents told me and my sister showed me that I could be anything I wanted to be. Through their support and insights, I have been able to achieve so much.

A final thank you to Adam and my dog, Raleigh, who have taught me so much about myself and motivate me every day to be a better person.

TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEDGMENTS iv

TABLE OF CONTENTS vi

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS x

CHAPTER 1 – THE α-CARBONIC ANHYDRASES 1

The α-carbonic anhydrase family members are structurally similar yet functionally diverse 1

Cytosolic CAs 2

Cytosolic carbonic anhydrase related proteins (CA-RPs) 5

Membrane-associated CAs 6

Mitochondrial CAs 9

Secreted CA 10

Using prior α-CA research to determine unknown functions of CAs 11

CHAPTER 2 – PREVIOUS RESEARCH ON CARBONIC ANHYDRASE III 16

Discovery of CA III 16

The CA III Conundrum 16

CA III knockout mice (Car3 KO) and controls respond similarly tocommon lab stressors 18

CA III and metabolism 19

CA III and oxidative damage 23

CA III expression drastically changes in response to many stimuli, but what is its function? 28

CHAPTER 3 – CARBONIC ANHYDRASE III (Car3) IS NOT REQUIRED FOR FATTY ACID SYNTHESIS AND DOES NOT PROTECT AGAINST HIGH-FAT DIET INDUCED OBESITY IN MICE 34

Summary 34

Introduction 35

Materials and Methods 38

Results 44

Discussion 48

Conclusion 51

CHAPTER 4 – CONCLUSIONS & FUTURE DIRECTIONS FOR CA III STUDIES 64

Conclusions 64

Potential confounding variables 65

Future directions 67

REFERENCES 77

LIST OF TABLES

Table 1.1. Localization, activity, and function of the α-CA family. 15

LIST OF FIGURES

Figure 1.1. CA activity is reliant on an effective proton shuttle and hydrophobic binding pocket. 13

Figure 1.2. Mitochondrial CA assists with ureagenesis, gluconeogenesis, and lipogenesis. 14

Figure 2.1. Rosiglitazone treated mouse and human adipocytes are smaller, more numerous, and have increased expression of Car3. 30

Figure 2.2. CA III expression decreases in many different liver diseases. 32

Figure 2.3. CA III is highly abundant and dynamically expressed in metabolic tissues 33

Figure 3.1. Amino acid sequence alignment of cytosolic carbonic anhydrases reveals key enzyme amino acids changes that affect activity. 53

Figure 3.2. 2D-DIGE identifies Car3 as a protein with decreased expression in livers of high-fat diet fed mice treated with Niclosamide. 54

Figure 3.3. Car3 is not required for de novo lipogenesis. 55

Figure 3.4. *Car3* -/- mice fed high-fat diet showed no difference in weight gain, food consumption, or body composition. 57

Figure 3.5. *Car3* -/- mice show no changes in total energy expenditure, RER, or activity. 59

Figure 3.6. *Car3* -/- does not affect high-fat diet-induced insulin-resistance. 61

Figure 3.7. *Car3* -/- mice show no difference in serum and tissue lipids and lipid substrates. 62

Figure 3.8. *Car3* -/- mice have normal thermoregulation. 63

Figure 4.1. CA III has no effect on oxidation of proteins in livers of mice. 74

Figure 4.2. Potential binding of CA with carboxyl groups of lipids. 75

Figure 4.3. Car3 -/- mice drink more water than WT mice when fasting. 76

LIST OF ABBREVIATIONS

2D-DIGE	2D-Differential Gel Electrophoresis
ACC	Acetyl-CoA carboxylase
ADP	Adenosine triphosphate
ATP	Adenosine triphosphate
ATP7B	Copper-transporting ATPase 2
AUC	Area under the curve
BAT	Brown adipose tissue
CA	Carbonic anhydrase
Ca	Calcium
CA III	Carbonic anhydrase III
CA-RP	Carbonic anhydrase related proteins
Car3	Carbonic anhydrase III mouse protein
CD36	Cluster of differentiation 36
Ci	Curie
CNS	Central nervous system
CO_2	Carbon dioxide
CPS1	Carbamoyl phosphate 1
Cys	Cysteine
Da	Dalton
DIO	Diet induced obesity
DMSO	Dimethyl sulfoxide
g	Gram

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosyl-phoshatiydyl-inositol
GSH	Glutathione
GTT	Glucose tolerance test
H^+	Proton
H_2CO_3	Carbonic acid
H_2O	Water
HCO_3^-	Bicarbonate
HIF-1	Hypoxia Factor 1
His	Histidine
IACUC	Institutional Animal Care and Use Committee
IP	Intraperitoneal
IP_3	Inositol 1,4,5-triphosphate
k_{cat}	Catalytic turnover
KO	Knockout
L	Liter
LD	Lipid droplet
Lys	Lysine
MEFs	Mouse embryonic fibroblasts
mRNA	Messenger RNA
NEFA	Non-esterified fatty acids
Nicl	Niclosamide
PC	Pyruvate carboxylase

PEP	Phosphoenolpyruvate
Phe	Phenylalanine
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
s	Second
SOD	Superoxide dismutase
STZ	Streptozotocin
T2D	Type 2 diabetes
TZD	Thiazolidinedione
UCP1	Uncoupling protein 1
WAT	White adipose tissue
WT	Wildtype
Zn	Zinc

CHAPTER 1 – THE α-CARBONIC ANHYDRASES

The α-carbonic anhydrase family members are structurally similar yet functionally diverse

Carbonic anhydrases (CAs) are metalloenzymes that catalyze the reversible carboxylation of water into carbonic acid, which dissociates into bicarbonate and protons:

$$H_2O + CO_2 \leftarrow\rightarrow H_2CO_3 \leftarrow\rightarrow HCO_3^- + H^+$$

Carbonic anhydrase II is one of the fastest enzymes known to man, with a k_{cat} of 1.4×10^6 s^{-1} (Supuran 2008). While carbon dioxide and oxygen can spontaneously interact to form bicarbonate, organisms in all life kingdoms use CAs to catalyze the reaction fast enough for buffering capabilities (Del Prete, Vullo et al. 2014). Carbonic anhydrase was originally identified for its necessary role in transporting CO_2 from tissues into the blood as bicarbonate (Meldrum and Roughton 1933). Since the discovery and isolation of the first carbonic anhydrase in 1933 (Meldrum and Roughton 1933), nearly 12,000 articles, reviews, and books have been written about carbonic anhydrases. Additionally since 1933, four evolutionarily distinct carbonic anhydrase gene families have been discovered: α-CA family (vertebrates), β-CA (bacteria, algae, and plants), γ-CA (archaea and some bacteria), and δ-CA (marine diatoms) (Supuran 2008). As of the completion of this manuscript, carbonic anhydrases are implicated in a large variety of functions: respiration, acid-base homeostasis, ion transport, taste preferences, ureagenesis, gluconeogenesis, and photosynthesis (Sly and Hu 1995) (Dodgson 1991, Shah, Rubbelke et al. 2013, Patrikainen, Pan et al. 2014) (Badger and Price 1994, Lehenkari, Hentunen et al. 1998).

The α-CA family is comprised mostly, 13 out of the 16 isozymes, of active enzymes that contain a functional catalytic site. These active CAs are structurally very similar especially near

the active site, which is comprised of three histidine (His) residues coordinated to Zn^{2+} (Figure 1.1) (Aggarwal, Boone et al. 2013). The rate-limiting step of carbonic anhydrase reactions is regenerating the active form of the enzyme, which requires a proton shuttle (Supuran 2008). Differences in amino acids near the active site, particularly around the shuttle lead to decreased carbonic anhydrase activity (Elder, Fisher et al. 2007).

There are 16 unique isozymes of carbonic anhydrase in mammals found in the α-CA family (Supuran 2008). In order to have such a wide range of physiological functions, the subcellular localization of these proteins is diverse: eight localize to the cytosol, five are transmembrane or membrane bound, two localize to the mitochondria, and one is secreted (Supuran 2008). The remainder of this chapter will discuss activity of CA isozymes, tissue localization, and proposed function for all of the mammalian carbonic anhydrases.

Cytosolic CAs

CA II is essential for respiration, CA I assists but is not required

CA I and CA II have a physiological role in transporting CO_2 generated by tissues to the lungs for expulsion. CO_2 diffuses across membranes into blood cells where both CA I and CA II hydrate the carbon dioxide into carbonic acid, which subsequently dissociates into protons and bicarbonate molecules. The protons bind to hemoglobin and bicarbonate diffuses out of the erythrocytes into the plasma for delivery to the lungs. Near the lungs, the reverse reaction occurs, and the bicarbonate from the plasma is converted back to CO_2 via the CAs in the red blood cell, and the CO_2 is expelled (Esbaugh and Tufts 2006, Frost 2014). In addition to red blood cells, CA I is located in the large intestine, eyes, sweat glands and adipose tissue, whereas CA II is expressed in at least one cell type of every organ/tissue (Sly and Hu 1995).

Even though CA I is the most abundant protein in erythrocytes after hemoglobin, it appears that CA II is more critical to maintaining cellular respiration (Edwards, Drummond et al. 2000). Humans deficient in CA II have systemic issues balancing pH and respiration, highlighted by issues maintaining homeostasis in the kidneys, brain and bones, whereas humans with CA I mutations do not develop a disorder (Sly and Hu 1995, Shah, Bonapace et al. 2004). The difference in disease outcome can be explained by the difference in activity of the two carbonic anhydrases; the activity of CA II, with the k_{cat} of 1.4×10^6 s^{-1} is about 85% higher than CA I, $k_{cat} = 2.0 \times 10^5$ s^{-1} (Supuran 2008). While other carbonic anhydrases or molecular pathways can compensate for a deficiency of CA I, CA II is required for normal function, likely because of its ubiquitous expression and high activity (Sly and Hu 1995).

CA III has low activity and localizes to metabolic tissues

CA III is the slowest active enzyme in the α-CA family, with a k_{cat} of 1.0×10^4 s^{-1}. (Supuran 2008). It is highly abundant in white adipose, brown adipose, skeletal muscle, and liver. CA III can be post-translationally modified via S-glutathionylation in response to oxidative damage (Harju, Bootorabi et al. 2013). Little research has been published about its functionality; the leading hypothesis is that it is involved in metabolism due to its abundance in metabolic tissues. Subsequent later chapters will discuss CA III in much greater detail.

CA VII is implicated in seizures, oxidative damage, and tissue specific cancers

CA VII is a cytosolic CA that has a high catalytic activity, k_{cat} of 9.5×10^5 s^{-1}, which is similar to the fastest recorded enzyme, CA II (Supuran 2008). CA VII is expressed in the colon, liver, skeletal muscle, and brain (Frost 2014). CA VII knockout (KO) mice have a normal life span, reproductive success, and no obvious deleterious phenotypes. However, when subjected to high temperatures, they are protected from seizures (Ruusuvuori, Huebner et al. 2013). The top

hypothesis for this finding was that CA VII is responsible for providing the bicarbonate necessary for the ion transport that generates synchronous neuronal excitation typically seen in seizures (Ruusuvuori, Li et al. 2004, Ruusuvuori, Huebner et al. 2013).

CAVII can also be S-glutathionylated, which is an early molecular response to oxidative damage. There are two cysteine residues that have been found to react with glutathione without affecting the activity of the enzyme (Truppo, Supuran et al. 2012). Additionally, it was found that CA VII protected against apoptosis in cells transfected with CA VII, indicating a possible role in oxidative damage (Monti, De Simone et al. 2017).

In cancer, involvement of CA VII is tissue specific. CA VII is highly expressed in a variety of brain tumors; patients with CA VII negative tumors have a higher survival rate than those with CA VII expressed (Bootorabi 2011). However, in colorectal carcinomas, there is a 4-fold decrease in CA VII mRNA expression, which correlates with disease progression and is also predictive of prognosis (Yang, Hu et al. 2015, Viikila, Kivela et al. 2016). CA VII's specific molecular function in cancer is currently unclear.

CA XIII has a moderate catalytic activity and is widely expressed

CA XIII has moderate catalytic activity, a k_{cat} of 1.5×10^5 s^{-1} (Supuran 2008). It is widely distributed in both humans and mice. Specifically, CA XIII can be found in the thymus, small intestine, spleen, prostate, ovary, colon, and testis in humans and spleen, lung, kidney, heart, brain, skeletal muscle, and testis in mice (Lehtonen, Shen et al. 2004). Despite being discovered more than a decade ago as a unique CA isozyme, the physiological function of CA XIII is still unknown.

Cytosolic carbonic anhydrase related proteins (CA-RPs)

CA-RPS are catalytically dead and implicated in central nervous function

The CA-related proteins, CA VIII, X, and XI, are catalytically dead. While they have a high degree of homology with other active carbonic anhydrases, they are missing one, two, or all of the histidines critical for zinc binding and anhydrase activity: His94, 96, and 119 (Nishimori, Vullo et al. 2013).

Despite the lack of any anhydrase activity, CA-RPs are still physiologically necessary. CA VIII knockout mice have no control of their body movement; their limbs have involuntary contractions that cause twisting movements that persist throughout their life span (Jiao, Yan et al. 2005). A single nucleotide polymorphism was found in CA VIII of humans, which causes mild retardation as well as difficulty walking (Turkmen, Guo et al. 2009). Interestingly, CA VIII acts as a binding protein to regulate motor function: CA VIII regulates Ca^{2+} release from Purkinje cells (or motor coordination cells) located in the cerebellum, by binding to the inositol 1,4,5-triphosphate (IP_3) receptor, blocking its affinity for IP_3 (Hirota, Ando et al. 2003). Less is known about CA X and XI, however both are expressed in different regions of the central nervous system in mice and humans (Okamoto, Fujikawa-Adachi et al. 2001, Aspatwar, Tolvanen et al. 2010). While their precise function is unknown, they have very recently been found to bind and potentially assist surface expression of neurexins, proteins essential for synaptic function (Sterky, Trotter et al. 2017). These findings indicate that CA-RPs have functional roles outside of the carbonic anhydrase catalytic activity and can bind other proteins.

CA XI and VIII have also been implicated in enhancing proliferation and growth of cancer cells in a variety of tissue types. CA XI and VIII are both up-regulated in gastrointestinal

cancer, and CA VIII is additionally up-regulated in lung and colorectal cancer (Morimoto, Nishimori et al. 2005, Aspatwar, Tolvanen et al. 2010).

Membrane-associated CAs

CA IV assists in respiration, sperm motility, and extracellular buffering of the CNS

CA IV is one of two GPI-anchored, glycosyl-phoshatiydyl-inositol, proteins in the α-CA family. It is also the only membrane-associated CA with high activity in humans, k_{cat} 1.1 x 10^6 s^{-1} (Supuran 2008). CA IV contributes to extracellular buffering in the central nervous system, and it has been shown to assist in bicarbonate and CO_2 transport in lung, kidney, and in isolated cells from other tissues (Shah, Ulmasov et al. 2005, Waheed and Sly 2014). However its role in respiration and pH buffering do not seem critical for survival; CA IV knockout mice are viable and have normal urinary pH (Shah, Ulmasov et al. 2005). CA IV KO mice do produce smaller litters, however this seems due to problems with sperm motility (Shah, Ulmasov et al. 2005). CA IV KO males have less motile and slower sperm than WT males, as bicarbonate is a known regulator of sperm motility (Wandernoth, Raubuch et al. 2010, Wandernoth, Mannowetz et al. 2015). CA IV has many different functions and it is unclear how they all relate to one another.

CA IX elevates during hypoxia and regulates gastric homeostasis

CA IX is a moderately active membrane-associated CA, k_{cat} = 3.8 x 10^5 s^{-1}, that localizes to stomach, colon and pancreas (Hilvo, Rafajova et al. 2004, Supuran 2008). Hypoxia Factor 1, HIF-1, is a transcription factor that can regulate CA IX expression. Hypoxia is important in the development of cancerous cells, as they grow rapidly and are unable to maintain an adequate oxygen supply from the current vascularization and require initializing their own anaerobic metabolism for energy requirements (Benej, Pastorekova et al. 2014). During hypoxic events, as in cancerous tumors without adequate blood flow, CA IX expression is elevated (Benej,

Pastorekova et al. 2014). CA IX is highly expressed in cancers of several tissues where CA IX is not typically expressed: renal, lung, cervical, ovarian, esophageal, and breast carcinomas (Kallio, Hilvo et al. 2010). While the function of CA IX in cancer is unknown, it is likely promoting the growth of cancer in hypoxic conditions.

Conversely, in stomach cancers, CA IX expression is decreased (Kallio, Hilvo et al. 2010). However, this is likely due to the function CA IX plays in maintaining homeostasis in the stomach. CA IX deficiency in mice caused gastric hyperplasia and atrophy of gastric mucosa, which was exacerbated by a high salt diet (Leppilampi, Karttunen et al. 2005), indicating that CA IX is involved in maintaining proliferative homeostasis of gastric tissues (Gut, Parkkila et al. 2002).

CA XII also elevates during hypoxia and is required for fluid homeostasis

CA XII is another moderately active membrane associated CA, k_{cat} of 4.2×10^5 s^{-1}, that localizes to the kidney and appears to have a role in regulating fluid homeostasis (Halmi, Lehtonen et al. 2004) (Supuran 2008). Humans with loss-of-function mutations in CA XII have a cystic fibrosis-like disease with elevated sweat chloride levels, failure to thrive, lung disease, and dry mouth/tongue (Hong, Muhammad et al. 2015, Lee, Vecchio-Pagan et al. 2016). The mechanism for CA XII regulating appropriate levels of sweat and other fluids is currently not understood, but may involve regulation of ion transport/cotransport.

CA XII is another hypoxia-induced membrane-associated protein that is important in cancers. CA XII is highly expressed in renal cell carcinoma, esophageal squamous cell carcinoma, and in chemo-resistant cells of colorectal cancer. (Tureci, Sahin et al. 1998, Kopecka, Campia et al. 2015, Kopecka, Rankin et al. 2016). In esophageal cancer, CA XII serves as a prognostic tool-patients with a higher expression of CA XII in tumorous cells had a significantly

lower survival rate (Kopecka, Campia et al. 2015, Kopecka, Rankin et al. 2016). Similar to CA IX, the function of CA XII in cancer is unclear, but likely involved in promoting growth of tumor cells in the hypoxia observed in tumors.

CA XIV promotes retinal light response and extracellular buffering in the brain

CA XIV also has moderate activity, k_{cat} of 3.1 x 10^5 s^{-1}, and has been found in isolated cell types in the brain, kidney, liver, colon, retina, heart, and skeletal muscles (Whittington, Grubb et al. 2004, Supuran 2008). The CA XIV knockout had a significant decrease in retinal light response, indicating that CA XIV plays an important functional role in signaling of the photoreceptors of the eye (Ogilvie, Ohlemiller et al. 2007). CA XIV also contributes to the buffering of the extracellular space in the brain (Shah, Ulmasov et al. 2005).

Humans and chimpanzees evolved without CAXV

CA XV is the most recently discovered CA and little is known about its function. It is the only α-CA found to date that is not expressed in humans or chimpanzees; instead, these organisms have orthologues of non-functional pseudogenes (3 in humans and 2 in chimpanzees) that contain frame-shifts, insertions, or point mutations. Initial phylogenetic studies suggest CA XV is closely related to CA IV. Like CA IV, CA XV is a GPI-anchored protein located on the plasma membrane and is predominantly localized to the kidney. The CO_2 hydration rate of human CA IV is 10-fold higher than mouse CA IV (Waheed and Sly 2014). It is likely that evolution equipped humans and chimpanzees with a much more efficient CA IV and they no longer required the moderate CA XV activity (k_{cat} of 4.7 x 10^5 s^{-1} in mice) for bicarbonate buffering in the kidney (Hilvo, Tolvanen et al. 2005, Supuran 2008, Waheed and Sly 2014). Evolutionarily, transcription and translation of an enzyme that would only moderately assist in

activity could not be justified, especially if there is another enzyme that catalyzes the same reaction faster.

Mitochondrial CAs

CA VA & VB are required for ureagenesis

There are two isoforms of the mitochondrial carbonic anhydrases, CA VA and CA VB, which until 1999 were considered the same isoform (Shah, Hewett-Emmett et al. 2000). Mitochondrial CAs have been implicated in providing the bicarbonate necessary for many metabolic pathways either directly (ureagenesis, gluconeogenesis) or indirectly (lipogenesis) (Figure 1.2) (Dodgson and Forster 1986, Lynch, Fox et al. 1995, Hazen, Waheed et al. 1996). In the mitochondria, carbamoyl phosphate 1 and pyruvate carboxylase require bicarbonate to catalyze the first committed steps of ureagenesis and gluconeogenesis (Lusty 1978, Jitrapakdee, St Maurice et al. 2008) Mitochondrial CAs are necessary to rapidly generate bicarbonate for any mitochondrial biochemical pathways, as bicarbonate cannot permeate the inner mitochondrial membrane (Arias-Hidalgo, Hegermann et al. 2016). Mitochondrial CA VA and VB indirectly affect lipogenesis, a cytosolic metabolic pathway, by regulating oxoaloacetate production through pyruvate carboxylase. Increasing or decreasing levels of oxoaloacetate allows regulation of citrate, which can then enter lipogenesis (Lynch, Fox et al. 1995, Hazen, Waheed et al. 1996, Jitrapakdee, St Maurice et al. 2008).

CA VA is located primarily in the liver, skeletal muscle, and kidney. It has a moderately active $k_{cat} = 2.9 \times 10^5\ s^{-1}$, whereas CA VB is located more ubiquitously and has a faster k_{cat} of $9.5 \times 10^5\ s^{-1}$(Shah, Hewett-Emmett et al. 2000, Supuran 2008). CA VA deficiencies in humans result in hyperammonemia, or increased ammonia levels in the blood (van Karnebeek, Sly et al. 2014). To identify individual contributions of CA VA and CA VB, single and double knockout mice

were generated (Shah, Rubbelke et al. 2013). CA VA knockout mice weighed less and had higher levels of ammonia than WT, whereas CA VB KO mice had no observable phenotype. However, the phenotypes in the CA V double knockout mice were more severe than the CA VA single KO, indicating that, while CA VB single knockout has no phenotype, it still contributes to ureagenesis and other metabolic functions causing the decreased weight and higher levels of ammonia (Shah, Rubbelke et al. 2013). This suggests that while CA VB can contribute to ureagenesis and gluconeogenesis, it likely has a different primary functionality.

Secreted CA

CA VI affects taste perception and can contribute to cavity formation in teeth

Carbonic anhydrase VI is an abundant protein expressed in the submandibular gland (Sly and Hu 1995) (Frost 2014). Considered one of the main proteins secreted into saliva, the concentration of CA VI is 6.8 ± 4.3µg/ml in humans (Patrikainen, Pan et al. 2014). CA activity was discovered in human saliva in 1939, however CA VI was not identified as the isozyme until 50 years later (Kivela, Parkkila et al. 1999). Human CA VI activity has a k_{cat} of 3.4 x 10^5 s^{-1} (Supuran 2008). Initial studies utilizing human populations to understand the function of CA VI were confounding. In human association studies, papers detailing taste perception contradicted each other as to whether CA VI polymorphisms affected bitter or salt perception (Padiglia, Zonza et al. 2010, Feeney and Hayes 2014). Mice deficient in CA VI were tested on taste preferences and were found to drink more quinine, a bitter flavor, and less salt, while maintaining the same amount of taste buds as their wildtype counterparts (Patrikainen, Pan et al. 2014), indicating that CA VI assists in taste perception.

Additionally early research into the oral physiology of CA VI, indicated that CA VI protected against the formation of caries, or cavities (Kivela, Parkkila et al. 1999). However later

studies contradicted this hypothesis; mice deficient in CA VI were less susceptible to cavities than wildtype counterparts and children known to have cavities had higher CA VI activities in their saliva than children without (Culp, Robinson et al. 2011, Frasseto, Parisotto et al. 2012). The history of research into the function of CA VI highlights the importance of knockout mice in studying CAs, and how varied the phenotypes and functions can be.

Using prior α-CA research to determine unknown functions of CAs

Carbonic anhydrases are a diverse set of isozymes with a wide variety of physiological functions, from respiration to taste perception. Table 1.1 summarizes localization, catalytic activity, and proposed function for all isozymes of the α-CA family. We can utilize this CA knowledge of functionality as well as experimental methodology to aim our research at less understood CAs like CA III—the slowest carbonic anhydrase.

For instance, mitochondrial CA VA and cytosolic CA III have overlapping tissue localizations. CA VA was found to provide the bicarbonate for mitochondrial metabolic pathways. Perhaps CA III provides bicarbonate for fatty acid synthesis, which occurs in the cytosol. Alternatively, CA VII and CA III can both be S-glutathionylated, and CA VII has been implicated in oxidative damage response. CA III could instead be assisting in response to damage, or it may contribute to both metabolism and oxidative damage. Many CAs have multiple seemingly unrelated functions, like CA IV-which functions in respiration, sperm motility, and extracellular buffering of the CNS,

Whatever the function of CA III, the evolution of CA XV to pseudogenes in humans and chimps suggests that if CA III had a duplicate function to one of the faster carbonic anhydrases, then CA III might be lost in evolution. This points to CA III having an alternative function

besides simply catalyzing the carboxylation of water. Since CA III has such a slow activity, it could instead act like the catalytically dead CA-RPs and bind proteins to alter function.

Studying the function of CAs is complex. Many carbonic anhydrases overlap in functionality and tissue localization so it is challenging to determine which CA is contributing to a specific function. Utilizing single and double knockout mice, researchers have been able to parse minor differences in carbonic anhydrases. This will help in guiding our research into understanding the function of CA III using a knockout mouse model in Chapter 3.

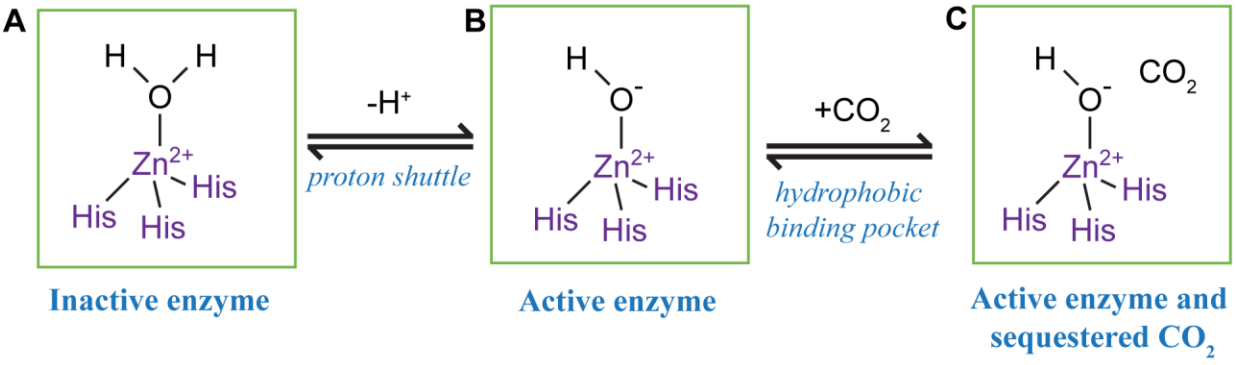


Figure 1.1. CA activity is reliant on an effective proton shuttle and hydrophobic binding pocket. (A) Active site of carbonic anhydrase, highlighted in purple, in the inactive form. (B) Under basic conditions, amino acids near the active site serve as proton shuttles that deprotonates the enzyme into the active form. (C) Carbon dioxide binds to a hydrophobic pocket near the active site and the reaction can begin. Adapted from (Supuran 2008).

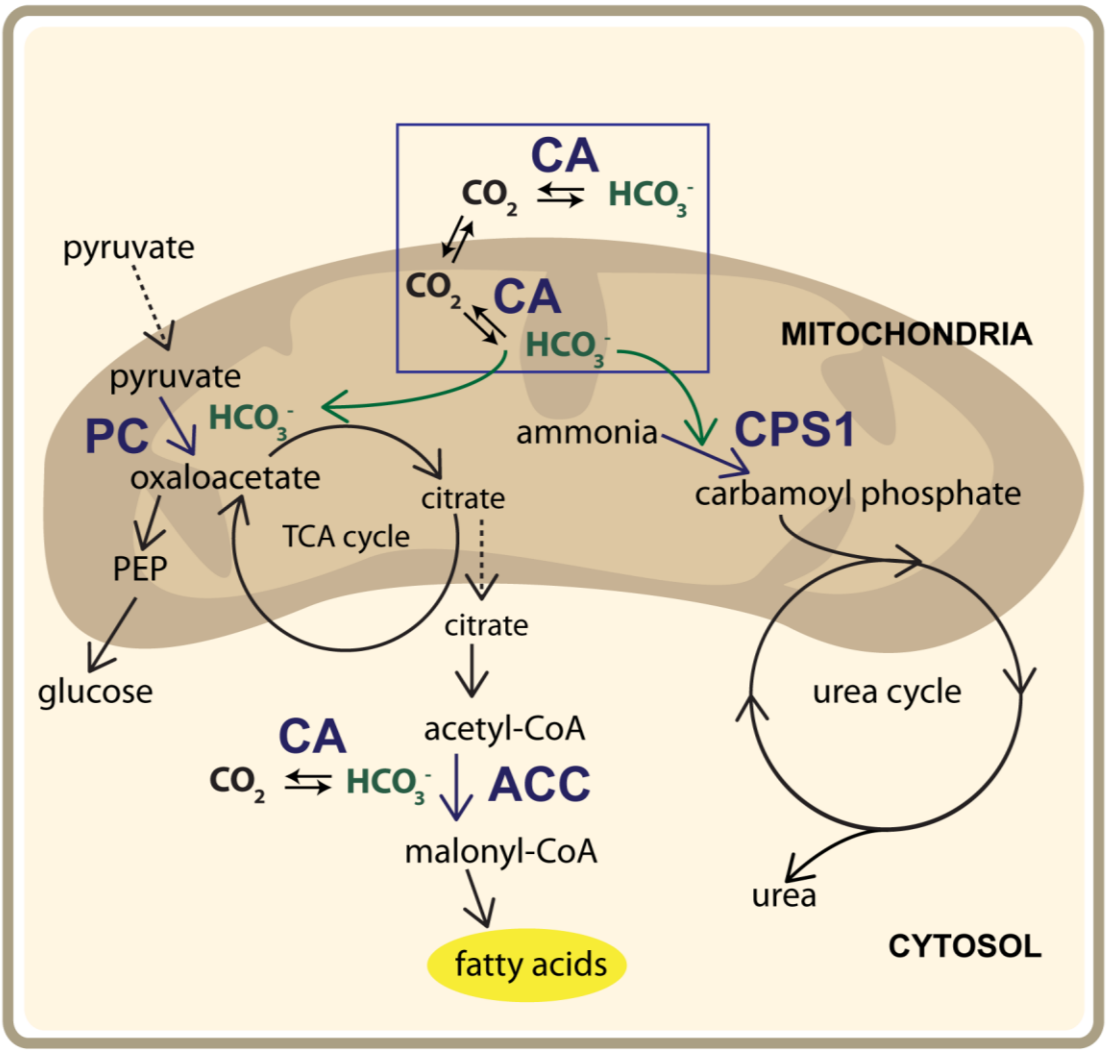


Figure 1.2. Mitochondrial CA assists with ureagenesis, gluconeogenesis, and lipogenesis. Mitochondrial and cytosolic CAs are required to provide bicarbonate to metabolic pathways. Inset blue box: Bicarbonate cannot diffuse through mitochondria. Abbreviations: PC- pyruvate carboxylase, HCO_3^-- bicarbonate, CA- carbonic anhydrase CPS1-carbamoyl phosphate 1, ACC-acetyl-CoA carboxylase, PEP- phosphoenolpyruvate. Adapted from (Dodgson and Forster 1986) (Hazen, Waheed et al. 1996) (Chegwidden, Dodgson et al. 2000) (Arias-Hidalgo, Hegermann et al. 2016).

Table 1.1. Localization, activity, and function of the α-CA family.

Intracellular localization	Isozyme	K_{cat} (x 10^5) (s^{-1})	Proposed functionality	Main Tissue localization
Cytosolic	I	2	• Respiration • Acid/base homeostasis	colon, eye, sweat glands, adipose tissue, blood cells
	II	14	• Respiration • Acid/base homeostasis	ubiquitous
	III	0.1	• Metabolism? • Oxidative damage?	adipose tissues, skeletal muscle, liver
	VII	9.5	• Seizures • Oxidative damage • Cancer	Brain, colon, liver, and skeletal muscle
	XIII	1.5	• unknown	lung, kidney, heart, brain, skeletal muscle, reproductive organs, intestines
Cytosolic (CA-RPs)	VIII	---	• Motor function • Proliferation of cancer	brain
	X	---	• CNS?	Central nervous system
	XI	---	• CNS? • Proliferation of cancer	Central nervous system
Membrane-associated	IV	11	• Respiration • Sperm motility • CNS buffering	lung, kidney
	IX	3.8	• Gastric homeostasis • Proliferation of cancer • Hypoxia?	stomach, colon, pancreas
	XII	4.2	• Fluid homeostasis • Proliferation of cancer • Hypoxia?	kidney
	XIV	3.1	• Photoreceptor signaling • CNS buffering	brain and eyes
	mXV	4.7	• Unknown	kidney
Mitochondrial	VA	2.9	• Ureagenesis • Gluconeogenesis • lipogenesis	liver, skeletal muscle, kidney
	VB	9.5	• Ureagenesis • Gluconeogenesis • lipogenesis	ubiquitous
Secreted	VI	3.4	• taste perception • cavities in teeth	salivary glands

CHAPTER 2 – PREVIOUS RESEARCH ON CARBONIC ANHYDRASE III

Discovery of CA III

CA III was originally identified as a unique carbonic anhydrase in 1978. The researchers who discovered CA III did not set out to study carbonic anhydrases, instead it was the "result of several years of frustrating search for the identity of an unknown protein" they found in their purification of phosphoglucose isomerase from skeletal muscle. CA III was a substantial by-product of their purification that they labeled "basic muscle protein", which they found "inconceivable at the time that a major component of solubilizable protein of skeletal muscle had not yet been identified" (Register, Koester et al. 1978). CA III is a very abundant protein in skeletal muscle comprising 1-2% of the total protein content (Register, Koester et al. 1978).

The CA III Conundrum

While CA III was originally identified in skeletal muscle of various animals, it was later found to be abundant in liver and adipose tissue of rodents (Shiels, Jeffery et al. 1984) (Spicer, Ge et al. 1990); CA III is highly abundant in these key metabolic tissues; it comprises 11% of the total protein and nearly 24% of cytosolic protein in rodent adipose tissue (Lynch, Brennan et al. 1993). Compared to the other active enzymes in the α-CA family, CA III has a very low carbonic anhydrase activity, with a k_{cat} ~1.0 x 10^4 s^{-1}, or roughly 0.16% of the activity of CA II, despite having a 62% sequence identity in amino acids in humans (Nishimori, Minakuchi et al. 2007) (Nishimori, Minakuchi et al. 2009). (Tu, Chen et al. 1994) While it is not catalytically dead, like the CA-RP proteins, it is the least active α-CA.

The low activity of CA III is caused by critical differences in amino acids near the active site's proton shuttle and hydrophobic binding pocket, Lysine 64 (K64) and Phenylalanine 198 (F198). In 1989, Tu *et al.* discovered a crucial amino acid in the proton shuttle of CA II. By making a CA II H64A mutant, they caused a disruption and the activity of CA II was greatly decreased. In CA II, the proton shuttle, which regenerates the active form of the enzyme, is much more efficient than in CA III because it has a histidine instead of a lysine. Tu *et al.* showed that it was due to proton shuttling by measuring the activity of H64A CA II mutants in solutions buffered with imidazole (the proton donor in histidine) and found that it was similar to wildtype (Tu, Silverman et al. 1989). By creating a K64H CA III mutant, Jewell *et al.* tested whether CA III activity could be enhanced to be as fast as CA II. They found mutated K64H CA III had higher activity than wildtype CA III, but not as high as CA II (Jewell, Tu et al. 1991).

To understand why CA III K64H was not as fast as CAII, more experimental mutations were made to CA II amino acids near the active site. The triple mutant CA III K64H R67N F198L, had a 500-fold increase in activity compared to wildtype CA III and had activities closer to wild type CA II (LoGrasso, Tu et al. 1991). Series of single and double mutants containing variants of K64, R67, and F198 allowed Tu *et al.* to determine that F198 was primarily responsible for the low activity of CA III (Tu, Chen et al. 1994). Through structural studies, it was determined that F198 in CA III resulted in a steric constriction near the hydrophobic binding pocket and thus altered interactions in the active site (Duda, Tu et al. 2005).

In the field, it is a conundrum as to why CA III is so abundant in tissues, when it appears to not be a very effective carbonic anhydrase. Nearly thirty years ago, Edsall wrote "it is a puzzle, however, that the tissue cells make use of such a weakly enzyme to do this job…'Why not the best?' –in other words, carbonic anhydrase II, which is present elsewhere in the body.

Apparently, the gene for enzyme III was there, it was adequate for the job it had to do, and there was no sufficient pressure that might have produced a switch to a higher activity enzyme" (Edsall 1984). It has often been thought that maybe instead of acting as a carbonic anhydrase, CA III has a different physiological function. The rest of the chapter will delve into experiments that have further developed our understanding of the function of CA III.

CA III knockout mice (Car3 KO) and controls respond similarly to common lab stressors

To identify an alternative physiological function of CA III, a knockout mouse was generated, which will be referred to as Car3 KO or Car3 -/-. The Kim *et al.* paper describes the generation of the Car3 KO mouse on a 129SvEv background and also tests a variety of common phenotypes-growth, development, effects of stress, and life span (Kim, Lee et al. 2004). Growth, life span, and reproductive success were normal in the Car3 deficient mice. Car3 WT and KO mice contained similar levels of free fatty acids in serum, muscle, liver, and adipose tissue. No significant changes in mRNA were measured in their brain, kidney, liver, soleus, or gastrocnemius microarrays when comparing wild type and Car3 deficient mice. Kim *et al.* speculated as to why they found no significant phenotype: "it may be that Car3 is required for an effective response to specific stimuli or stresses which mice do not encounter in the laboratory animal facility" (Kim, Lee et al. 2004). Additionally, the 129SvEv background may have been problematic for discovering differences in metabolic phenotypes as this background is less likely to become glucose intolerant or develop fatty livers (Biddinger, Almind et al. 2005) (Champy, Selloum et al. 2008). Even after the generation of a KO mouse line, the function of CA III is still unknown. The next two sections of this chapter break down the knowledge of CA III into two focal points of past research: metabolism and oxidative damage.

CA III and metabolism

CA III abundance decreases during starvation but increases when refed

CA III protein and gene expression seem highly nutritionally regulated; it is down-regulated in nutrient poor conditions and elevated in nutrient rich states. CA III gene expression is reduced in the muscle of starving mice after 24 hours by 1.7-fold, and further decreases to a 2.4-fold reduction after 48 hours (Jagoe, Lecker et al. 2002). Even more striking is CA III mRNA expression in liver decreased 10-20-fold, after a 24 hour fast, and close to 60-fold decrease following a 48-hour starvation (Zhang, Xu et al. 2011) (Bauer, Hamm et al. 2004). Other nutritionally deficient conditions, protein depletion in the diet and chronic alcohol consumption, also decreased the expression of CA III protein and mRNA (Ronchi, Conde et al. 2004) (Sanllorenti, Rosenfeld et al. 2001) (Caballero, Mendieta et al. 2011, Aroor, Roy et al. 2012) (Kharbanda, Vigneswara et al. 2009, Yamada, Satoh et al. 2013, Carter, Vigneswara et al. 2015). When refed after starvation, CA III mRNA levels increased to roughly half of the fed condition (Bauer, Hamm et al. 2004), which indicates that CA III responds to shifting dietary conditions and increases in nutrient-rich states. CA III protein and mRNA in both liver and white adipose tissue (WAT) also increased in rodents fed a high fat diet (Benard, Lim et al. 2016) (Bondia-Pons, Boque et al. 2011) (Mendez, Ciordia et al. 2017) (Van Schothorst, Franssen-van Hal et al. 2005).

CA III expression correlates with changes in insulin

The difference in CA III expression under fed/fasted states correlates with changes in insulin, a metabolic hormone critical to maintaining glucose homeostasis. Insulin and CA III expression are both elevated during the fed state and decreased under starvation (Bauer, Hamm et al. 2004). Additionally, in rats treated with streptozotocin, (STZ) a toxic drug targeted to

pancreatic islets that eliminates insulin production, CA III protein expression in liver decreased and CA III expression remained at normal levels when rats were given insulin after the STZ treatment (Nishita, Igarashi et al. 1995). This result implies that CA III expression responds to changes in insulin. Similarly, primary adipocytes treated with insulin increased CA III mRNA expression (Alver, Ucar et al. 2004). In nutrient rich states, insulin increases and stimulates the generation of new fatty acids in the cytosol. Because insulin and CA III expression are correlated, CA III could be providing the necessary bicarbonate for *de novo* fatty acid synthesis (Gibson, Titchener et al. 1958).

CA III expression is lower in adipose tissue and livers of obese rodents

Counter to the notion that CA III expression is high during nutrient rich state, there are rodent models of obesity that have decreased CA III expression compared to their lean siblings (Stanton, Ponte et al. 1991). While performing proteomic analysis to determine differences in *ob/ob* mice, Stanton *et al.* discovered that obese mice have decreased CA III protein and mRNA in WAT (Stanton, Ponte et al. 1991). Further proteomic analysis of other models of obesity, Zucker rats and NZO mice, confirmed that CA III decreases in WAT and liver of obese rodents (Lynch, Mccall et al. 1992) (Takahashi, Oh-Ishi et al. 2001) (Lynch, Brennan et al. 1993). However obese rodents still responded to starvation; in both lean and obese rodents, CA III mRNA decreased in WAT during fasting and increased upon refeeding (Stanton, Ponte et al. 1991), indicating a role for CA III unique to the rodent models. These rodent models of obesity have impaired leptin signaling, a hormone secreted from adipose that assists in modulating food intake and energy expenditure (Wang, Chandrasekera et al. 2014) (Friedman and Halaas 1998). In addition to gaining weight, rodent models of obesity can also model type 2 diabetes (T2D) as they have dyslipidemia, mild hyperglycemia, and insulin resistance (Wang, Chandrasekera et al.

2014). This could indicate a role for CA III downstream of the leptin pathway or other pathways involved in obesity/T2D.

CA III increases in adipose tissue of rosiglitazone treated mice

T2D is characterized by impaired glucose homeostasis and insulin resistance (Lai, Chandrasekera et al. 2014). Rosiglitazone is a thiazolidinedione (TZD) drug used to treat diabetes by increasing insulin sensitivity. TZDs are agonists to the nuclear receptor, PPAR-γ, which regulates adipocyte differentiation as well as lipid storage. TZDs increase insulin sensitivity by promoting differentiation of adipocytes, fatty acid uptake, and decreasing the amount of lipids stored in an individual adipocyte. In TZD-treated patients and rodents, adipocytes are smaller and more numerous compared to untreated (Figure 2.1) (Johnson, Trasino et al. 2007, Quinn, Hamilton et al. 2008). Previous work from the Spiegelman group has shown that CA III mRNA increased in WAT of mice fed a high-fat diet and treated with rosiglitazone. As expected with rosiglitazone treatments, these mice gained weight and body fat, yet became more insulin sensitive (Choi, Banks et al. 2010) (Choi, Banks et al. 2011). This change in CA III abundance in response to rosiglitazone could be due to many different parameters- the decrease in insulin, the increase in adipocyte differentiation or fatty acid uptake, the increase in overall weight and body fat, or a response to elevated PPAR-γ.

CA III may regulate adipogenesis through PPAR-γ

To investigate the role of CA III in adipogenesis, Mitterburger *et al.* isolated primary, mouse embryonic fibroblasts, MEFs, from the Car3 WT and KO mice and differentiated them into adipocytes. CA III protein expression increased in the wildtype primary adipocytes during differentiation, however, in the knockout MEFs, the cells differentiated more quickly, had higher triglycerides and ~1000-fold increase in PPAR-γ mRNA. These results indicated that CA III can

regulate differentiation of adipocytes and lipid storage by directly or indirectly lowering PPAR-γ expression (Mitterberger, Kim et al. 2012). However, in 3T3 cell lines, a fibroblast cell line, cells transfected with CA III grew exponentially faster than the control transfections, indicating CA III was contributing to, not inhibiting, the growth of these cells (Raisanen, Lehenkari et al. 1999). The relationship between CA III and cell growth is unclear as there are few but conflicting studies. Further research using *in vivo* models could help better elucidate a role for CA III in adipogenesis and lipid accumulation.

Skeletal muscles of Car3 knockout mice respond more slowly to stimuli

Muscle fatigue data from Car3 WT and KO mice further contributed indirect evidence to the hypothesis that CA III has a role in metabolism. The act of generating the mechanical energy necessary to move muscles requires ATP. ATP is initially generated in response to stimuli via phosphocreatine that is stored in locations that readily need access to quick sources of ATP: skeletal muscles, heart, and brain. Phosphocreatine donates its phosphate group to ADP in the surrounding muscle to quickly generate ATP (Wallimann, Wyss et al. 1992). After these short-term stores of phosphocreatine are depleted, after 10-20 seconds, glycolysis, oxidative phosphorylation, and fatty acid oxidation are required to generate the ATP required to move muscles (Sahlin, Tonkonogi et al. 1998). In response to stimulation, Car3 WT and KO muscles reached the same levels of maximum recovery, however Car3 KO mice had a slower initial rate (Liu, Walter et al. 2007, Feng and Jin 2016). Real time analysis of phosphocreatine, ATP, and free phosphates in the muscles implied that while both Car3 WT and KO mice eventually generated enough ATP for maximal recovery, the Car3 KO mice had to utilize phosphocreatine ATP stores for slightly longer than WT mice. This implied that Car3 KO mice had impaired

regulation of alternative ATP producing pathways: glycolysis, oxidative phosphorylation, or fatty acid synthesis (Sahlin, Tonkonogi et al. 1998, Liu, Walter et al. 2007).

CA III decreases during cold stress when fatty acid oxidation is high

In addition to maintaining body temperature in cold conditions, brown adipose tissue (BAT), has the ability to take up and store fatty acids and glucose (Townsend and Tseng 2014). BAT has been implicated in maintaining a healthy weight and preventing metabolic disease (Harms and Seale 2013). During cold exposure, brown adipose tissues increase lipolysis of triglycerides and fatty acid oxidation to generate heat (Harms and Seale 2013). CA III mRNA decreased after prolonged exposure to cold temperatures in BAT of mice (Walden, Hansen et al. 2012) (Forner, Kumar et al. 2009). This decrease in CA III in response to prolonged exposure in cold temperatures, hints at roles opposing fatty acid oxidation and promoting fatty acid uptake, lipid synthesis, or storage of lipids.

CA III and oxidative damage

CA III is S-glutathionylated in response to oxidative damage

S-glutathionylation is a post-translational modification of the amino acid cysteine, where the sulfhydryl group of the cysteine binds to the sulfur-containing compound, glutathione (GSH). This modification is an early defense against oxidative damage in cells (Thomas, Poland et al. 1995, Dalle-Donne, Rossi et al. 2009). In 1991, investigators were trying to identify the highly abundant, 30 kDa protein that was susceptible to S-glutathionylation in response to oxidative stress (Chai, Jung et al. 1991). They purified and identified the protein as CA III and determined that CA III has 3 forms: reduced, one cysteine S-glutathionylated, and two cysteines S-glutathionylated. They were able to visualize the separate protein forms by using isoelectric focusing and incubating CA III with glutathione, to induce glutathionylation, or glutathione

disulfide, to reverse the glutathionylation (Chai, Jung et al. 1991). It was later confirmed by crystallography that two cysteine residues, 181 and 186, are both susceptible to S-glutathionylation in CA III (Mallis, Poland et al. 2000) (Kim and Levine 2005).

Zimmerman *et al.* studied levels of S-glutathionylation in skeletal muscle of mice subjected to oxidative damage. By restricting blood flow and oxygen to the muscle of anesthetized mice, they found that CA III is increasingly S-glutathionylated during ischemic conditions (Zimmerman, Wang et al. 2004) Since CA III can be S-glutathionylated during oxidation, researchers have proposed CA III has a function in cellular response to oxidative damage (Dalle-Donne, Rossi et al. 2009).

Superoxide dismutase KO mice have lower CA III in liver

Superoxide dismutase (SOD) helps to protect cells against oxidative damage by quickly converting the harmful superoxide into peroxide, which can be broken down by other enzymes (Elchuri, Oberley et al. 2005). Younger SOD KO mice show no overt phenotypes, however, SOD KO mice have a shorter life span and their livers are more susceptible to many types of oxidative damage and spontaneously develop tumors (Elchuri, Oberley et al. 2005). While doing proteomic studies on SOD KO mice, Elchuri *et al.* determined that CA III protein and mRNA expression were lower in livers of SOD knockout mice and expression decreased with age (Elchuri, Naeemuddin et al. 2007). Additionally, they found that the amount of oxidized CA III protein was lower in SOD KO mice (Elchuri, Oberley et al. 2005). This further indicated that CA III could be involved in an oxidative damage response.

Copper accumulation in the liver leads to decrease in CA III expression

Wilson's disease is a disorder in humans caused by mutations in a copper transporter that results in copper accumulations in the liver, brain, and eyes (Fuentealba and Aburto 2003).

Copper accumulation in the liver leads to oxidative damage through inflammation and fibrosis of the liver (Fuentealba and Aburto 2003). Both Wilson's disease and a Wilson's disease mouse model are characterized by an accumulation of copper in the liver; in mutant livers of mice, copper accumulates to about 800µg/g of liver, compared to 12µg/g in WT animals (Grimes, Paynter et al. 1997). Grimes *et al.* found CA III protein and mRNA levels decreased in the livers of these mutant mice. They also found CA III mRNA levels decreased in WT mice in response to copper injections, which confirmed the decrease in CA III expression was due to copper accumulation (Grimes, Paynter et al. 1997).

CA III abundance decreases with hepatoxins associated with fatty liver disease

While CA III abundance decreases in response to many different liver diseases (Figure 2.2), these three different hepatoxic events –alcohol, protein deficiency, and carbon tetrachloride, can all be used to induce fatty liver disease in mice (Yamada, Satoh et al. 2013, Scholten, Trebicka et al. 2015, Kitade, Chen et al. 2017). Fatty liver disease progression has three main stages: accumulation of fat in the liver, inflammation and oxidative stress response to excess fat, fibrosis and cirrhosis of the liver (Kitade, Chen et al. 2017). The role of CA III in fatty liver diseases is not well understood; however, it could be contributing to the lipid dysregulation of the liver, the oxidative response to fat accumulation or both.

CA III is one of the most down-regulated proteins and mRNAs in rodents fed alcohol, it decreased nearly 5-fold in the livers of rats chronically fed alcohol (Aroor, Roy et al. 2012) (Kharbanda, Vigneswara et al. 2009, Yamada, Satoh et al. 2013, Carter, Vigneswara et al. 2015). Chronic alcohol consumption can lead to liver damage through up-regulation of inflammation and oxidative stress, which eventually leads to steatosis and cirrhosis of the liver (Yamada, Satoh

et al. 2013). A decrease in CA III in response to chronic alcoholism implies that CA III may be a protectant against inflammation and steatosis as it decreases as liver damage increases.

Protein depletion in the diet causes weight loss and susceptibility to infection and oxidative damage. Superoxide dismutase, a key protein that responds to oxidative damage, is down-regulated in the liver of mice during protein depletion, which leaves the liver vulnerable to oxidative damage. (Ronchi, Conde et al. 2004) (Sanllorenti, Rosenfeld et al. 2001). Protein depletion in mice decreased the total amount of all proteins in liver by 50%. CA III protein decreased by ~40% after protein depletion, and is one of the highest down-regulated proteins (Sanllorenti, Rosenfeld et al. 2001, Caballero, Mendieta et al. 2011).

The decline in CA III in protein free diet fed mice can be rescued by supplementing with either methionine or cysteine (Ronchi, Conde et al. 2004). Methionine and cysteine are both sulfur-containing amino acids that can be involved in oxidative pathways as well as the development of fatty livers in mice (Brosnan and Brosnan 2006, Toohey 2014). Cysteine can often be a limiting factor for glutathione synthesis, a key antioxidant involved in S-glutathionylation of proteins like CA III (Brosnan and Brosnan 2006). Cysteine is derived from methionine, so it is possible that the rescue of the CA III expression is due to the availability of cysteine and cysteine precursors (methionine) in the diet (Brosnan and Brosnan 2006), which allows for more S-glutathionylation of CA III and oxidative damage response.

All hepatoxic events discussed so far have pointed to CA III being down-regulated during times of oxidative stress, however some hepatotoxic compounds like acetaminophen, amiodarone hydrochloride, tetracycline hydrochloride and carbon tetrachloride acutely increased protein expression of CA III in rodent livers 24 hours after treatment (Yamamoto, Kikkawa et al. 2006). Yet, with prolonged exposure of carbon tetrachloride or large doses of acetaminophen,

CA III protein expression decreased; there was a 20-fold decrease of CA III in rats after 4 days of exposure to acetaminophen (Henkel, Roderfeld et al. 2006, Wong, Fan et al. 2011, Eakins, Walsh et al. 2015). This implies that CA III protein expression increases immediately in response to oxidative stress, but as it responds it is degraded quickly or is only necessary for the initial response to injury.

CA III overexpression protects against apoptosis in cells subjected to oxidative damage

Hypoxia, the deficiency of oxygen in tissues, and hydrogen peroxide, a common reactive oxygen species, can trigger oxidative damage to cells and tissues, which can result in the induction of apoptosis (Shang, Bao et al. 2012) (Raisanen, Lehenkari et al. 1999). In C2C12 and 3T3 cell lines, CA III was overexpressed and oxidative stress was induced using either hypoxia or hydrogen peroxide (Shang, Bao et al. 2012) (Raisanen, Lehenkari et al. 1999). In both experiments, CA III overexpression significantly lowered the rate of apoptosis and in the 3T3 cell line, the intracellular oxidation levels were lower in CA III transfected cells. This indicated that overexpression of CA III corresponded with lower levels of cell death possibly by protecting the cells from oxidative damage (Shang, Bao et al. 2012) (Raisanen, Lehenkari et al. 1999).

Car3 KO mice were not affected by oxidative damage

While CA III appears to protect against oxidative damage induced apoptosis in cell culture, Car3 deficiency *in vivo* does not affect oxidative damage. Hyperoxia, excess oxygen in tissues, induces oxidative damage. Car3 WT and KO mice were exposed to a 100% oxygen environment to induce hyperoxia and oxidative damage, no difference was found in survival between the genotypes (Kim, Lee et al. 2004). Car3 deficient soleus muscles, a slow-twitch skeletal muscle, behaved the same as wildtype muscles when subjected to a hypoxia challenge.

Both groups of mice had a similar internal pH, indicating the response to oxidative damage is not affected by Car3 deficiency (Liu, Walter et al. 2007).

CA III expression drastically changes in response to many stimuli, but what is its function?

Even before its discovery as a unique isozyme, CA III has been "frustrating" researchers for years, either as an unknown contaminant or now as a puzzling protein without a defined function (Register, Koester et al. 1978). Much of what is know about CA III's role originates from proteomic studies of metabolic or oxidative damage stressors. From this research, we can glean that CA III expression changes in response to many different stressors (Figure 2.3), suggesting a physiological function in different molecular pathways.

CA III is abundant in metabolic tissues that can store fat: liver, skeletal muscle and adipose tissues. Prior research implies a role for CA III in various pathways related to lipid metabolism: *de novo* lipogenesis, fatty acid uptake, lipid accumulation leading to fatty liver disease, and fatty acid oxidation. While dynamic regulation and tissue localization of CA III may suggest a novel function of CA III, the relationship between CA III and lipid metabolism has never directly been tested.

CA III can also be post-translationally be modified via S-glutathionylation and consequently it has been suggested that CA III assists in the initial reaction to protect cells and tissues from oxidative damage. Accordingly, CA III expression responded to prolonged exposure of multiple stressors that promote oxidative cell damage. CA III also protected against oxidative damage induced apoptosis *in vitro*, however, *in vivo* studies with CA III KO mice indicated CA III was not critical for oxidative damage response. The conflicting *in vitro* and *in vivo* results makes the involvement of CA III in oxidative damage unclear.

Despite being discovered nearly 40 years ago, the function of the slowest CA, CA III, remains an enigma. The next chapter discusses how we further tested the role of CA III in metabolism *in vivo*.

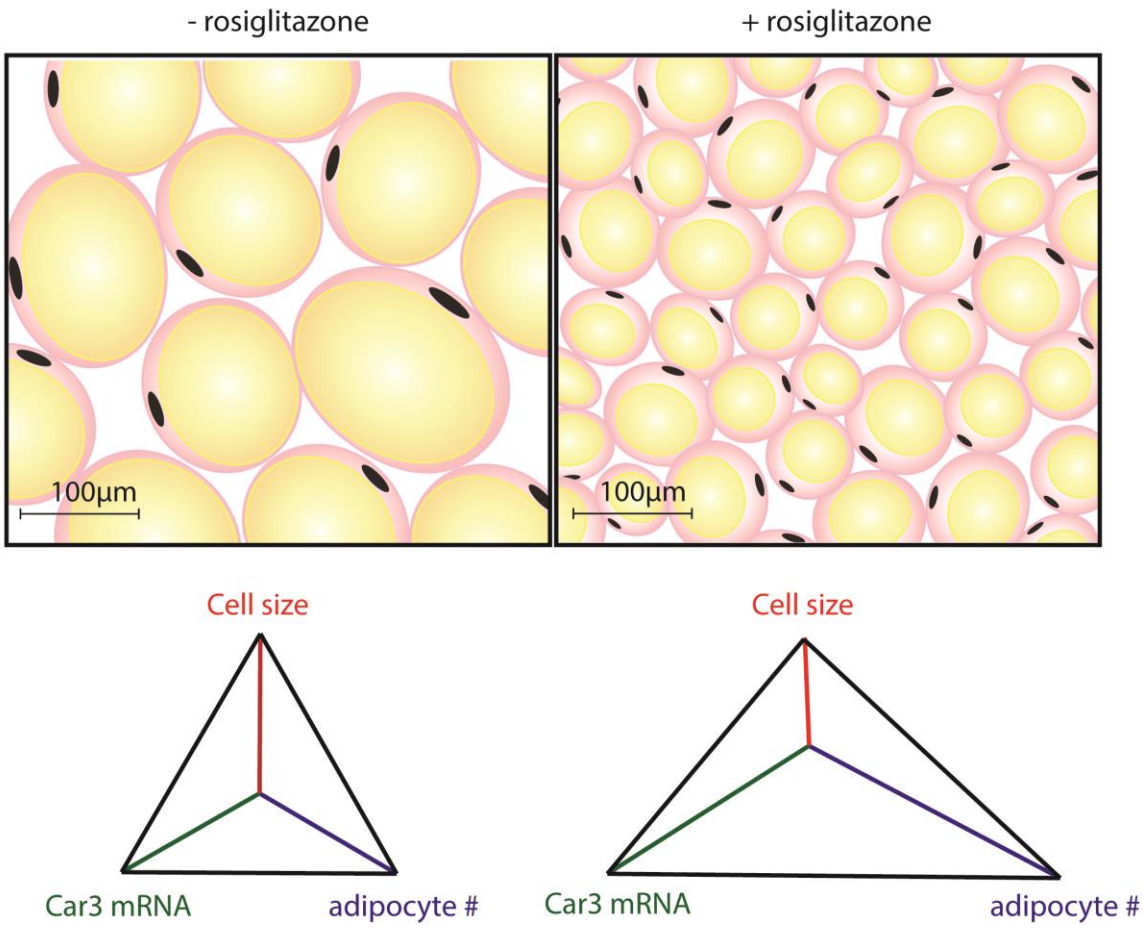
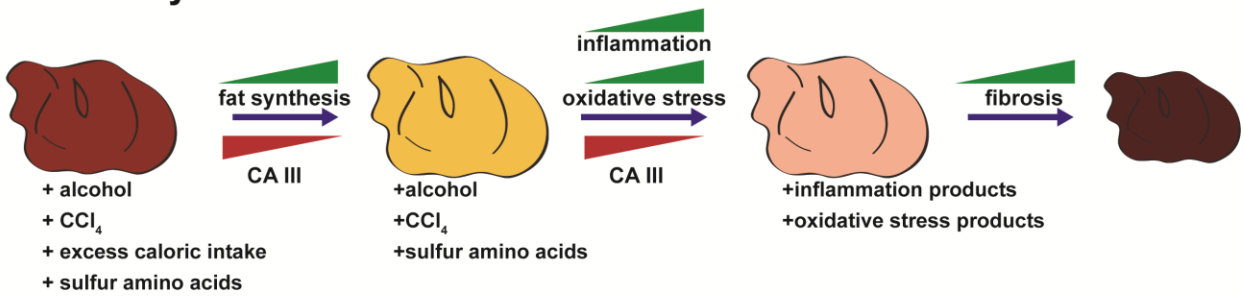


Figure 2.1. Rosiglitazone treated mouse and human adipocytes are smaller, more numerous, and have increased expression of Car3. Top panel: Adipocyte morphology in untreated and treated diabetic mouse and human patients. Bottom panel: Graphical representation of key traits in rosiglitazone untreated and treated adipocytes. Adapted from (Johnson, Trasino et al. 2007) (Choi, Banks et al. 2010) (Choi, Banks et al. 2011).

A.

Fatty Liver Disease

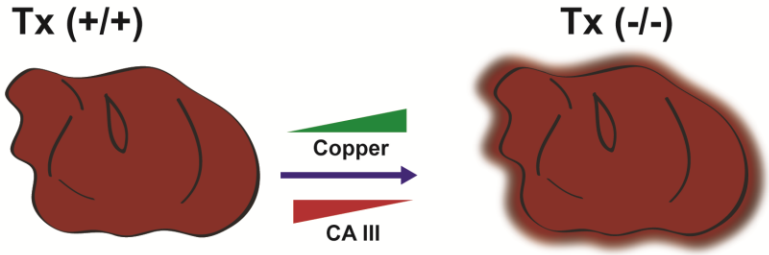


Disease Phenotypes:

progression of fatty liver disease ends with necrosis of the liver and death

B.

Wilson's Disease

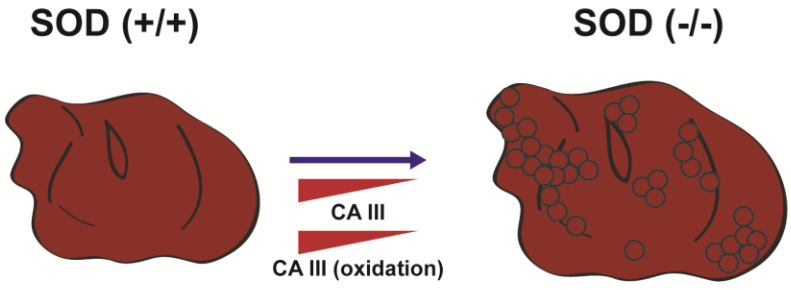


Disease Phenotypes:

Inflammation of liver, Neural Fibrosis of liver

C.

Liver Cancer



Disease Phenotypes:

Increased oxidative damage, more lipid peroxidation, more oxidized proteins, tumoregenesis, decreased lifespan.

Figure 2.2. CA III expression decreases in many different liver diseases. (A) Progression of fatty liver disease can be triggered through many stressors, and follows a two-hit model. (B) Wilson's Disease in mice is caused by the mutation in copper transporter Tx. (C) Liver cancer can be modeled in SOD knockout mice as they develop tumors as they age. Adapted from (Kitade, Chen et al. 2017) (Anstee and Goldin 2006) (Grimes, Paynter et al. 1997, Fuentealba and Aburto 2003, Elchuri, Oberley et al. 2005).

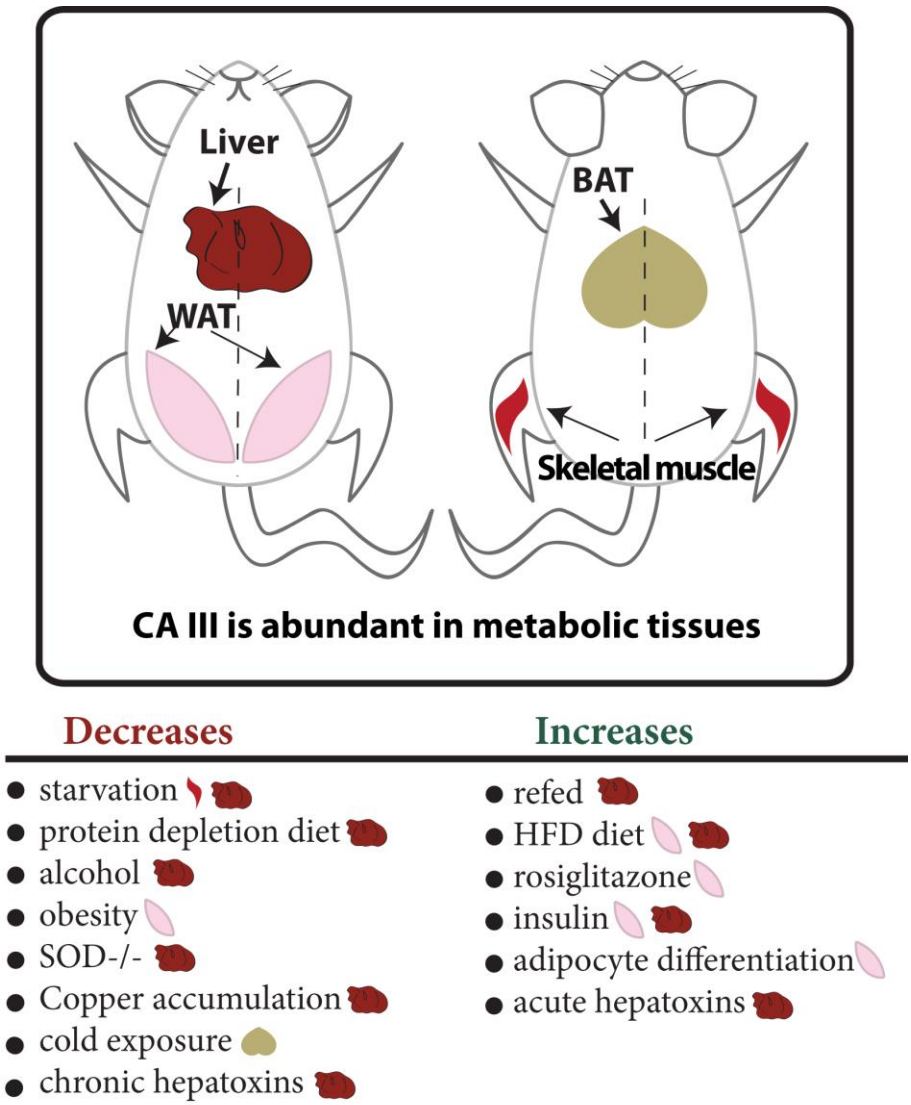


Figure 2.3. CA III is highly abundant and dynamically expressed in metabolic tissues CA III is abundant in liver, white adipose tissue (WAT), brown adipose tissue (BAT), and skeletal muscle. CA III mRNA and protein expression changes in response to many different stimuli.

CHAPTER 3 – CARBONIC ANHYDRASE III (Car3) IS NOT REQUIRED FOR FATTY ACID SYNTHESIS AND DOES NOT PROTECT AGAINST HIGH-FAT DIET INDUCED OBESITY IN MICE[1]

Summary

Carbonic anhydrases are a family of enzymes that catalyze the reversible condensation of water and carbon dioxide to carbonic acid, which spontaneously dissociates to bicarbonate. Carbonic anhydrase III (Car3) is nutritionally regulated at both the mRNA and protein level. It is highly enriched in tissues that synthesize and/or store fat: liver, white adipose tissue, brown adipose tissue, and skeletal muscle. Previous characterization of Car3 knockout mice focused on mice fed standard diets, not high-fat diets that significantly alter the tissues that highly express Car3. We observed lower protein levels of Car3 in high-fat diet fed mice treated with niclosamide, a drug published to improve fatty liver symptoms in mice. However it is unknown if Car3 is simply a biomarker reflecting lipid accumulation or whether it has a functional role in regulating lipid metabolism. We focused our *in vitro* studies toward metabolic pathways that require bicarbonate. To further determine the role of Car3 in metabolism, we measured *de novo* fatty acid synthesis with *in vitro* radiolabeled experiments and examined metabolic biomarkers in Car3 knockout and wild type mice fed high-fat diet. Specifically, we analyzed body weight, body composition, metabolic rate, insulin resistance, serum and tissue triglycerides. Our results

[1] This chapter previously appeared as an article in PLoS One. The original citation is as follows: Renner, S. W., L. M. Walker, L. J. Forsberg, J. Z. Sexton and J. E. Brenman (2017). "Carbonic anhydrase III (Car3) is not required for fatty acid synthesis and does not protect against high-fat diet induced obesity in mice." PLoS One **12**(4): e0176502.

indicate that Car3 is not required for *de novo* lipogenesis, and Car3 knockout mice fed high-fat diet do not have significant differences in responses to various diets to wild type mice.

Introduction

Carbonic anhydrases (CAs) are isozymes that catalyze the carboxylation of water into carbonic acid, which spontaneously dissociates into bicarbonate and protons ($H_2O + CO_2$ $\leftarrow H_2CO_3 \rightarrow HCO_3^- + H^+$) (Meldrum and Roughton 1933, Lindskog and Silverman 2000). There are 16 unique isozymes in the vertebrate carbonic anhydrase gene family, or the α-CA family (Supuran 2008). Carbonic anhydrases are involved in a wide variety of functions: respiration, acid-base homeostasis, ion transport, bone resorption, taste preferences, ureagenesis and gluconeogenesis (Dodgson 1991, Sly and Hu 1995, Lehenkari, Hentunen et al. 1998, Shah, Rubbelke et al. 2013, Patrikainen, Pan et al. 2014). The subcellular localization of these proteins is diverse: eight localize in the cytosol, five are transmembrane or membrane bound, two localize to the mitochondria, and one is secreted (Supuran 2008). The α-CA family is comprised of active enzymes that contain a functional catalytic site (CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV, XV) as well as catalytically dead enzymes known as carbonic anhydrase related proteins (CA-RP VIII, X and XI) (Supuran 2008). While most of the active carbonic anhydrases have some of the highest activity rates in biology, CA III has 0.16% of the activity compared to CA II (Chegwidden and Carter 2000, Nishimori, Minakuchi et al. 2009). Unlike the CA-RP proteins, which have mutated histidine (His) residues in the active catalytic site leaving them enzymatically inactive, CA III still maintains the histidine site for CAs, containing a zinc molecule surrounded by three His residues (Nishimori, Minakuchi et al. 2007, Supuran 2008, Aspatwar, Tolvanen et al. 2014). However, CA III does differ in amino acid alignment with the closely related CA I and CA II proteins at two important residues, as depicted in (Figure 3.1),

lysine 64 (Lys) and phenylalanine 198 (Phe), which dramatically decrease the activity of CA III (LoGrasso, Tu et al. 1993, Tu, Chen et al. 1994, An, Tu et al. 2002, Duda, Tu et al. 2005, Elder, Fisher et al. 2007). While the function of CA III is currently unknown it has been speculated to be involved in metabolism, oxidative damage response, and mitochondrial ATP synthesis (Raisanen, Lehenkari et al. 1999, Alver, Ucar et al. 2004, Zimmerman, Wang et al. 2004, Liu, Walter et al. 2007, Mitterberger, Kim et al. 2012)

CA III (Car3 in mice) is highly abundant in tissues that can store lipids in mice: liver, brown fat and white adipose tissues (approximately 24% of soluble protein in white adipose), and slow-twitch skeletal muscle (Lynch, Brennan et al. 1993). Car3 appears to be nutritionally regulated; during starvation Car3 mRNA decreases in rodent livers (Dodgson, Quistorff et al. 1993, Bauer, Hamm et al. 2004, Zhang, Xu et al. 2011) and Car3 mRNA subsequently increases upon refeeding. Car3 protein expression also increases in rodents fed Western-type high fat diets in both livers (31-83% increased protein) and white adipose tissue (97-129% increased mRNA) (Van Schothorst, Franssen-van Hal et al. 2005, Bondia-Pons, Boque et al. 2011, Mendez, Ciordia et al. 2017) Additionally, dietary stressors on rodents, including protein depletion and alcohol consumption, decrease the mRNA and protein levels of Car3 in the liver. (Sanllorenti, Rosenfeld et al. 2001, Ronchi, Conde et al. 2004, Kharbanda, Vigneswara et al. 2009, Caballero, Mendieta et al. 2011, Aroor, Roy et al. 2012, Yamada, Satoh et al. 2013, Carter, Vigneswara et al. 2015). Car3 expression also changes in response to insulin, a key metabolic hormone. Car3 mRNA expression increases in response to insulin in adipose tissue (Alver, Ucar et al. 2004) and Car3 protein decreases in liver when insulin is decreased in rats through injection of streptozotocin (Dodgson and Watford 1990, Nishita, Igarashi et al. 1995). Since *de novo* lipogenesis occurs during nutrient-rich states when insulin levels are high (Mabrouk, Helmy et al. 1990), and there

is the requirement of bicarbonate substrate in the committed step of fatty acid synthesis, acetyl-Coa → malonyl-CoA, (Gibson, Titchener et al. 1958), we hypothesized that carbonic anhydrase III is providing the necessary bicarbonate for *de novo* lipogenesis. CAs that are highly enzymatically active have been suggested to provide the bicarbonate for *de novo* lipogenesis; animals and cells with pharmacologically decreased CAs have decreased *de novo* lipogenesis (Herbert and Coulson 1984, Lynch, Fox et al. 1995, Hazen, Waheed et al. 1996), but the role of Car3, with much lower enzymatic activity, in *de novo* lipogenesis has not been previously studied.

In rodent models of diabetes/obesity, Car3 protein expression in liver and/or adipose tissue decreases in obese Zucker rats and in *ob/ob* mice compared to wild type rodents (Stanton, Ponte et al. 1991, Lynch, Mccall et al. 1992, Lynch, Brennan et al. 1993). Even though Car3 may decrease, acetyl Co-A carboxylase (ACC) protein expression is up-regulated in adipose tissue of obese Zucker rats (Lynch, Mccall et al. 1992), perhaps contradictory with a role for Car3 in *de novo* lipogenesis.

The small molecule drug Niclosamide (Nicl) has recently been implicated as a potential therapeutic for type 2 diabetes (T2D) and fatty liver disease in mice fed HFD (Tao, Zhang et al. 2014, Chowdhury, Turner et al. 2017). Niclosamide prevents insulin resistance in high-fat diet fed mice, and improves insulin sensitivity in *db/db* mice, a leptin signaling deficient rodent model (Tao, Zhang et al. 2014, Wang, Chandrasekera et al. 2014). Excess storage of lipids in liver, adipose, and muscle tissues contributes to T2D, and mice treated with niclosamide have reduced fat accumulation in liver and decreased liver triglycerides in a fatty liver mouse model (Tao, Zhang et al. 2014). In this study, we used two-dimensional differential gel electrophoresis (2D-DIGE) to identify Car3 as a protein highly expressed in DIO mice with fatty liver, but

down-regulated in DIO mice treated with niclosamide. This result suggests that Car3 may reflect the lipid content of tissues.

To determine whether Car3 has a functional role in lipid content we characterized the previously described *Car3 -/-* mice (Kim, Lee et al. 2004). No obvious deleterious phenotypes have been reported in previous studies on Car3 knockout mice; however, the affect of specialized diets on these knockout mice was not previously researched (Kim, Lee et al. 2004, Zimmerman, Wang et al. 2004, Liu, Walter et al. 2007, Mitterberger, Kim et al. 2012, Feng and Jin 2016). In this study, we investigate Car3 function in *de novo* lipogenesis by measuring fatty acid synthesis in isolated mouse hepatocytes, and during induced obesity through high-fat diet by measuring subsequent changes in body weight, dyslipidemia, insulin resistance, food intake, and energy expenditure.

Materials and Methods

2D-DIGE protocol and western blot confirmation animal Care

20-week old diet-induced obesity (DIO) mice were purchased from Jackson Labs, with mice starting on the high-fat diet (D12492, Research Diets) at 6 weeks. At 20 weeks of age, mice were treated with 10mg/kg niclosamide or DMSO vehicle control for 12 days by daily intraperitoneal (IP) injections. Mice were harvested to obtain liver tissue. Mice were maintained at the University of North Carolina (UNC) under protocols specifically approved for this study by the UNC Institutional Animal Care and Use Committee (IACUC).

2D-Differential Gel Electrophoresis (2D-DIGE) of niclosamide-treated mouse liver

Liver lysates were prepared, and 2D-DIGE experiments performed by the University of North Carolina Systems-Proteomics Center core facility as previously described (Osorio, Sullivan et al. 2007, Onyenwoke, Forsberg et al. 2012). Protein spots were identified using

peptide mass fingerprinting tandem mass spectrometry data by the Yale Mass Spectometry and Proteomics Resource Core (New Haven, CT), according to previously described methods (Osorio, Sullivan et al. 2007, Pinaud, Osorio et al. 2008, Onyenwoke, Forsberg et al. 2012).

Western blotting of mouse liver lysates

20 week old DIO high-fat diet fed mice were treated for 12 days with either 5 mg/kg, 25 mg/kg niclosamide, or DMSO (vehicle) by daily IP injections (40 µl). On the last day of treatment, mice were sacrificed and livers harvested and frozen on dry ice. For western blot analysis, liver samples were homogenized and sonicated in 10 volumes of RIA lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 1 mM beta-glycerolphosphate, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1X Sigma-Aldrich P8340 protease inhibitor cocktail). Homogenates were clarified by centrifugation at 16,000xg for 10 minutes at 4°C, and protein in the supernatant was quantified using the Bio-Rad Dc Assay (Hercules, CA). Protein samples in loading buffer (Life Technologies NP0007) were loaded at 50 µg per well on 4-12% NuPAGE gels and separated for 45 minutes at 200V, then transferred to Immobilon-FL blotting membrane at 300 mA for 1 hour. Blots were blocked with 5% milk in TBS, then probed for carbonic anhydrase III (Santa Cruz Biotechnology, sc-50715) and subsequently actin (MP Biomedicals, #69100) to control for total protein loaded. Secondary antibodies were (IRDye infrared antibodies, LI-COR Biosciences, Lincoln, NE) (Onyenwoke, Forsberg et al. 2012). The Odyssey Infrared Imaging System was used for quantification of the relative intensity ratio of Car3/Actin (LI-COR Biosciences, Lincoln, NE).

Animal Care for Car3 knockout mice

Car3 -/- mice were obtained from NIH (kind gift from Dr. Levine) on a mixed 129SvEv background as previously generated (Kim, Lee et al. 2004). To outcross the *Car3* knockout alleles into a C57BL/6J background more appropriate for metabolic studies (Almind and Kahn 2004, Andrikopoulos, Massa et al. 2005, Berglund, Li et al. 2008), male progeny were backcrossed with C57BL/6J female at least 8 generations. Sibling matched males were used in all analyses, unless otherwise specified. Mice were weaned at 3-4 weeks of age and placed on standard chow (2020X, Teklad Diets, Madison WI). Mice were maintained at the University of North Carolina under protocols specifically approved for this study by the UNC IACUC.

Hepatocyte isolation and de novo lipogenesis assays

Hepatocytes were isolated using a protocol adapted from Wendel *et al*. (Wendel, Cooper et al. 2013). Littermate female mice fed standard chow were anesthetized by IP injection of 250mg/kg tribromoethanol. Once mouse toes were unresponsive to touch, a 24G catheter was inserted into the portal vein. The liver was perfused with Perfusion Buffer 1 (115mM NaCl, 25mM HEPES, 5mM KCl, 1mM KH_2PO_4, 525µM EGTA, pH 7.4) warmed to 37°C, and then Perfusion Buffer 2 (115mM NaCl, 25mM HEPES, 5mM KCl, 1mM KH_2PO_4, 2.5mM $MgSO_4$, 1.6mM $CaCl_2$, 1.4-1.8mg/ml collagenase Type I (Worthington Chemicals, Lakewood, NJ), pH 7.4) warmed to 37°C. After perfusion was complete, the liver was excised and hepatocytes were released by gently tearing the liver lobes in chilled Isolation Medium, DMEM (Gibco #31053-028), 5% Fetal Bovine Serum, 1% Penicillin/Streptomycin, 2mM L-Glutamine, 1x MEM Non-Essential Amino Acids, 4µg/ml insulin, 0.1µM Dexamethasone. The hepatocytes were then filtered through a 100µm cell strainer and centrifuged at 50 x *g* for 2 min at 4°C. The cells were washed twice with cold Isolation Medium, and resuspended in warmed Isolation Medium for cell

counting and plating. Hepatocytes were plated on collagen-coated 6-well dishes with $1x10^6$ cells per well, and incubated at 37°C with 5% CO_2. After cells attached to the plates (4 h post-plating), media were changed to warmed L-15 media with 1% penicillin/streptomycin. Sixteen to 20 h after plating, hepatocytes were rinsed with warmed 1x PBS, (Gibco #14190-144) and then treated with 2ml of Assay Medium, L15 media (Gibco #11415-064), 1% penicillin/streptomycin, 0.8μCi/ml [1,2-^{14}C] acetic acid (Perkin Elmer Life Sciences, NEC553001MC) for 2 hours. The hepatocytes were rinsed with warmed 1x PBS, and then lysed with methanol and scraped. Lipids were extracted from hepatocyte lysates using water:chloroform:methanol (0.8:2:2 v/v/v). Lipids from the chloroform layer were collected, dried down, and counted in scintillation vials. Protein quantifications were made using duplicate plates from the same hepatocyte isolation. Cells were scraped and sonicated in SDS lysis buffer (2% SDS, 60mM Tris-Cl pH 6.8, 1mM PMSF, 2.5 mM sodium pyrophosphate, 1mM beta-glycerolphosphate, 1mM sodium orthovanadate, 1X Sigma-Aldrich P8340 protease inhibitor cocktail). Cell lysates were clarified by centrifugation at 16,000xg for 10 minutes at 4°C, and protein in the supernatant was quantified using the Bio-Rad Dc Assay (Hercules, CA).

Metabolic characterization of mice on high fat diet

At 5-6 weeks of age, mice either remained on standard chow or were placed on a high-fat diet (Almind and Kahn 2004, Winzell and Ahren 2004, Wang and Liao 2012). The standard chow caloric consumption is 24 kcal% protein, 16 kcal% fat, and 60 kcal% carbohydrate, and the energy density is 3.1 kcal/g food (2020X, Teklad Diets, Madison WI). The high-fat diet caloric consumption is 20 kcal% protein, 60 kcal% fat, and 20 kcal% carbohydrate, and the energy density is 5.24 kcal/g food (D12492, Research Diets, New Brunswick, NJ).

Metabolic chambers and body composition

Mouse body composition, energy expenditure, activity, and food intake were measured at the Animal Metabolism Phenotyping Core at UNC under the supervision of Dr Kunjie Hua. Body composition was determined by MRI at 11 weeks of age (EchoMRI, Houston, TX) (Nixon, Zhang et al. 2010). Energy expenditure, activity, and food intake were measured at 13-14 weeks of age using CaloCages (PhenoMaster, TSE systems, Chesterfield, MO). Data were collected for 2 days; the first day was considered the acclimation period and was excluded from data analysis (Cooper, Grevengoed et al. 2015). Mice had unlimited access to food and water for the entire duration in metabolic chambers.

Serum measurements

Fasted mice were starved overnight (15-16 hours) (Champy, Selloum et al. 2004) and measurements were taken at the beginning of the light cycle. To better synchronize the feeding schedule for fed measurements, food was removed from the cages during the light cycle, and mice were fed *ad libitum* during the dark cycle. Fed measurements were taken at the beginning of the light cycle after three nights of entrainment as described (Wang, Quagliarini et al. 2013). Glucose was measured with a drop of whole blood using a glucometer (Bayer Contour, Leverkusen, Germany). Whole blood was collected from mice using Microvette® tubes (CB300Z, Sarstedt, Nümbrecht, Germany) under fasting and fed conditions. To extract serum from whole blood, the blood was allowed to clot for 30-90 min at room temperature and then centrifuged at 2000 x *g* for 20 min (4°C), per provided instructions from Microvette. Insulin levels were determined using a mouse insulin ELISA kit (Crystal Chem Inc, Downers Grove, IL). Serum triglycerides (Wako Diagnostics, Richmond, VA), non-esterfied fatty acids (Wako

Diagnostics, Richmond, VA), cholesterol (Stanbio, Boerne, TX) and glycerol (Sigma-Aldrich, St. Louis, MO) were determined with enzymatic colorimetric assays.

Oral glucose tolerance test (GTT)

Sixteen-week-old mice were fasted overnight for 14-15 hours followed by oral gavage of 2 mg/g body weight of glucose. Blood glucose was measured at 0, 15, 30, 60, and 120 minute time points using a glucometer, as described previously described (Ayala, Samuel et al. 2010, Deol, Evans et al. 2015).

Tissue triglycerides

At 18 weeks of age, mice were sacrificed and tissues were weighed and collected for further analysis. Liver triglycerides were measured from the left lobe, while muscle triglycerides were measured from calf muscle using a protocol adapted from (Zagani, El-Assaad et al. 2015). Tissues were homogenized on ice using the BeadBug (Benchmark Scientific, Edison, NJ) with 50-100 mg of tissue in 10 or 20 volumes of 5% NP-40 in water (10 volumes for liver samples and 30 volumes for muscle samples). Samples were boiled @ 100°C for 5 min, then cooled to room temperature twice. The samples were then centrifuged at 16,000 x *g* for 2 min to pellet denatured DNA and proteins. Supernatants were then used to measure triglycerides using a colorimetric assay (Sigma-Aldrich, St. Louis, MO).

Body temperature and thermoregulation

Body temperatures from mice fed standard or high-fat diet were measured by rectal probe under fasting (15-18 hours) and fed (*ad libitum*) conditions (MicroTherma 2, ThermoWorks, American Fork, UT) (Dubuc, Wilden et al. 1985). Adult female mice, 8-13 weeks of age, were fasted overnight and exposed to 4°C for 5 hours (Putri, Syamsunarno et al. 2015).

Statistics

All data and statistics were analyzed using GraphPad Prism 7. Data are expressed as mean ± SD. Significance was determined using Tukey's multiple comparisons for all figures except for body weights, energy expenditures, RER, GTT area under the curve (AUC), and tissue triglycerides. For GTT AUC and tissue triglycerides, significance was determined using unpaired T-test. A significance threshold of $P < 0.05$ was applied. For body weight, energy expenditure, and RER, discovery was determined by multiple T-tests using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1% (Benjamini, Krieger et al. 2006). Each row was analyzed individually, without assuming consistent SD.

Results

Niclosamide treated mice have lower Car3 protein expression

In previous unpublished experiments, we observed that mice with fatty liver induced by diet-induced obesity (DIO) and treated with niclosamide had lower liver triglycerides and improved insulin sensitivity compared to untreated mice, which has been independently confirmed (Tao, Zhang et al. 2014). In an effort to identify which proteins might change in response to niclosamide treatment, we used 2D-DIGE analyses to compare liver protein expression from high-fat DIO treated with niclosamide or vehicle. Spots were then analyzed for differences in expression. One of the most abundant differentially expressed spot on the 2D-DIGE gel was excised and identified as Car3 (Figure 3.2A). In order to verify that Car3 protein expression changed with niclosamide treatment, we performed western blots on liver lysates from additional DIO mice treated with vehicle, 5 mg/kg or 25 mg/kg body weight doses of niclosamide (Figure 3.2B). The western blots confirmed the 2D-DIGE results with high and low doses of niclosamide leading to lowered Car3 protein levels (standardized to actin) compared to the vehicle-treated mice (mean relative Car3 intensities of 1.56, 2.16 and 2.54 respectively).

Car3 is not required for de novo fatty acid synthesis

Given the correlation between Car3 expression and fatty liver we wondered whether Car3 plays a role in lipid homeostasis. Given that bicarbonate is required for lipid synthesis, we tested the hypothesis that Car3 might be required for lipogenesis, using the radiolabeled-acetate incorporation assay to compare *de novo* fatty acid synthesis of wild type and *Car3-/-* primary mouse hepatocytes. Because the rate-limiting step of fatty acid synthesis requires bicarbonate (Figure 3.3A), we were concerned that traditional cell culture incubations utilizing a bicarbonate-based buffering system would mask any potential differences in [1,2-^{14}C]acetate incorporation into lipid. To account for this, we used L-15 media, a cell culture media with a bicarbonate-free buffering system, to culture and assay the hepatocytes. We also performed the assay in the presence and absence of carbon dioxide during incubation. For both with or without CO_2, the incorporation of [1,2-^{14}C]acetate into lipids was statistically indistinguishable between wild type and *Car3 -/-* cells. (Figure 3.3B) The incorporation of [^{14}C]acetate into fatty acids was 65-67% higher in the hepatocytes incubated with 5% CO_2, as expected, since that increases free bicarbonate in the media available for the first step in fatty acid synthesis (Figure 3.3A). These results indicate that Car3 expression has no significant affect on *de novo* lipogenesis in primary liver cells. Liver is the primary site of *de novo* lipogenesis in mammals.

Weight gain, body composition, and chow consumption is not effected by Car3

To determine if Car3 affects body weight or composition, wild type and *Car3 -/-* mice were put on a high-fat diet (HFD) or standard chow at 5 weeks of age and measured once a week for 10 weeks. Body weights of wild type and *Car3 -/-* mice did not differ significantly at any age for either standard chow or high-fat diet (Figure 3.4A). After 8-9 weeks on the diets, food consumption was monitored over a pre-acclimated 24-hour period in a metabolic chamber

(Figure 3.4B). For both diets, Car3 did not effect food consumption. Wild type and *Car3 -/-* mice ate the same amount of standard chow or HFD during both the light and dark cycle. To see if fat and lean mass were individually affected, we measured body composition on standard chow and high-fat diet. The lean masses of wild type and *Car3 -/-* mice were not statistically different, with the overall mean at 22.85 ± 1.058g (Figure 3.4C). Similarly, fat masses of wild type and *Car3 -/-* mice were not significantly different, with overall mean fat mass on standard chow at 1.031 ± 0.3721 and on high-fat diet at 7.729 ± 2.358 (Figure 3.4C). Mice on HFD had an increased contribution of fat mass compared to the mice on standard chow, as expected. These results suggest that Car3 is not affecting weight gain or fat and lean mass composition under ad libitum feeding conditions.

Car3 has no significant affect on total energy expenditure or energy substrate utilization with standard chow or HFD fed mice

To determine if Car3 affects fuel source utilization, we measured total energy expenditure, energy substrate usage, and physical movement activity in metabolic chambers. Wild type and *Car3 -/-* mice on both diets showed no difference in activity or energy expenditure (Figure 3.5A). To measure contribution of lipids or carbohydrates to energy usage, respiratory exchange ratios (RER) were calculated for the wild type and *Car3 -/-* mice fed standard chow and HFD; an RER equal to 0.7 indicating fatty acid oxidation is the main source of energy, whereas an RER equal to 1.0 or greater indicates carbohydrates are the primary fuel source. Wild type and *Car3 -/-* mice have similar RER values throughout the 24-hour monitoring period, and the light and dark cycle values were not statistically different for either standard chow or HFD-fed mice (Figure 3.5B). During both the light and dark cycle, there were also no significant differences in movement activity/average beam breaks for wild type and *Car3 -/-* mice fed standard chow or HFD (Figure 3.5C).

Car3 has no effect on HFD-induced insulin resistance

Mice fed high-fat diets can have elevated fasting blood glucose and insulin reflecting lower insulin sensitivity (Wang and Liao 2012, Eisinger, Liebisch et al. 2014). To test the effect of Car3 on insulin sensitivity, we performed glucose tolerance tests (GTT) and collected fasting blood glucose and insulin on mice fed either standard chow or high-fat diet. Since liver Car3 mRNA expression is highest in the fed state, to maximize possible differences between wild type and knockout mice, we also obtained fed blood measurements (Bauer, Hamm et al. 2004). Both wild type and *Car3 -/-* mice fed standard chow had lower blood glucose and insulin in the fasting state than in the fed state (as expected); similarly HFD-fed mice had elevated fasting blood glucose and insulin compared to standard chow fed mice (Figures 3.6A and 3.6B), as also expected. Glucose tolerance tests in mice fed standard chow and high-fat diet were not statistically significant between wild type and *Car3 -/-* mice in individual glucose measurements or area under the curve calculations (Figure 3.6C).

Car3 knockouts have normal serum and tissue lipid/fatty acid biomarker levels

To see if Car3 plays a role in increased liver triglycerides found in mice with fatty liver, we measured serum and tissue lipids (fatty acids and triglycerides). As stated previously, fed serum measurements were also obtained due to Car3 mRNA expression being highest in the liver in the fed state. Fasted and fed serum triglycerides, non-esterfied fatty acids (NEFA), cholesterol, and glycerol levels were all measured and found not statistically different between wild type and *Car3 -/-* mice on either diet (Figures 3.7A-3.7D). Liver and muscle triglyceride content also showed no difference between wild type and *Car3 -/-* mice (Figures 3.7E and 3.7F).

Is Car3 required for lipid uptake/mobilization during thermogenesis?

Given the abundance of Car3 in adipose and skeletal muscle and increase in fatty livers, this was somewhat reminiscent of the expression of CD36 protein, which also increases in fatty liver and is abundant in adipose tissue (Koonen, Jacobs et al. 2007, Sheedfar, Sung et al. 2014). One of the most profound phenotypes of CD36 knockout mice is the inability to maintain core body temperature under cold challenge due to suspected inability to uptake lipid and subsequently hydrolyze it for energy/heat, particularly in brown fat (Bartelt, Bruns et al. 2011). Given the similarity in expression of CD36 and Car3 proteins in lipid-storing tissues, we subjected *Car3 -/-* mice to cold temperature challenge. *Car3 -/-* mice are not impaired in their ability to maintain core body temperature in the cold (Figure 3.8).

Discussion

Despite the abundance of Car3 in key metabolic tissues including white and brown fat, liver, and skeletal muscle its function remains unknown. The relatively high abundance of Car3 these tissues– in fats Car3 is nearly 30% of soluble protein and 8% in slow-twitch muscle (Lynch, Brennan et al. 1993, Kim, Lee et al. 2004)– led to extensive analysis of *Car3 -/-* muscle function but showed relatively mild muscle phenotypes with normal "growth, development and life span" (Kim, Lee et al. 2004).

Although the exact function of Car3 remains unknown, many researchers postulate Car3 function is not as a carbon dioxide hydratase given its relatively miniscule enzymatic activity compared to Car1 and Car2 (0.16% with purified protein *in vitro* (Nishimori, Minakuchi et al. 2009)). Additional evidences suggesting Car3 may not function to produce bicarbonate *in vivo* are observations that the most potent Car3 inhibitor is carbonate ion (10μM K_I), which is in equilibrium with bicarbonate itself (Nishimori, Minakuchi et al. 2009). These investigators

stated “it is difficult to explain why Nature preserved … such a “bad” catalyst” that is also most potently inhibited by a proposed equilibrium product.

Even though evidence suggests Car3 may have no functional role in producing bicarbonate, we still explored potential roles for Car3 in lipid homeostasis because Car3 protein is highly enriched in tissues that can accumulate or synthesize lipid, and because bicarbonate is essential for fatty acid synthesis (Alver, Ucar et al. 2004). The three prior studies characterizing Car3 knockout mice did not investigate phenotypes related to lipid synthesis or storage nor did these studies feed high-fat diets to find any phenotypes (Kim, Lee et al. 2004, Liu, Walter et al. 2007, Feng and Jin 2016).

Although Car3 may not drive bicarbonate production *in vivo*, no one previously used *Car3 -/-* mice to demonstrate that Car3 is not required for *de novo* lipogenesis *in vivo* (Fig 3). Given the abundance of Car3 in lipid containing tissues, we decided to focus on the response of *Car3 -/-* mice fed a high-fat diet known to alter lipid homeostasis. We measured key metabolic indicators including: weight gain, body composition, chow consumption, energy expenditure, insulin resistance, and dyslipidemia. *Car3* deficiency had no affect on the biomarkers we tested, suggesting that Car3 is not responsible for the synthesis of fat nor does it affect the mobilization of fat in these tissues.

We identified Car3 as a protein increased in the liver in a mouse model fed high-fat diet but lowered with niclosamide (Fig 2), a drug shown to improve fatty liver and T2D-like symptoms (Tao, Zhang et al. 2014). Since Car3 liver protein expression was higher in DIO mice fed HFD, and lower in niclosamide treated HFD-fed DIO mice, we hypothesized that Car3 was involved in the development of fatty liver in this disease model. We saw no difference in liver triglycerides of wild type and *Car3 -/-* mice fed HFD, which suggests that Car3 is not required

for accumulation of fat in the liver. While Car3 could be a biomarker for fatty liver disease in DIO mice, it is also possible that the effect of decreased Car3 seen in niclosamide-treated mouse livers may simply reflect lower fat content in the tissue, without regulating its decrease.

The high abundance of Car3 in selected tissues and relative lack of enzymatic activity suggest perhaps Car3 plays a non-enzymatic role. There is evidence that Car3 acts as a protectant against oxidative damage; cells transfected with Car3 are protected from apoptosis induced by hydrogen peroxide (Raisanen, Lehenkari et al. 1999). Car3 can also undergo the posttranslational modification by S-glutathionylation at two cysteine residues, Cys186 and Cys181 not found in Car1/2 (Chai, Jung et al. 1991, Lii, Chai et al. 1994, Mallis, Poland et al. 2000). S-glutathionylation of proteins is an early response to oxidative damage (Thomas, Poland et al. 1995). S-glutathionylation of Car3 increases with increased oxidative damage that occurs naturally as in aging (Cabiscol and Levine 1995) or with muscle damage (Zimmerman, Wang et al. 2004). Car3 contains numerous other additional cysteine residues not found in Car1/2. The concept of Car3 as an abundant "scavenging" protein to protect against oxidative damage is becoming an area of more research focus (Monti, De Simone et al. 2017).

Interestingly, Car3 protein expression is altered in SOD1, copper zinc super oxide dismutase 1, providing another connection to oxidative stress responses in tissues. In *SOD1 -/-* mouse knockouts Car3 protein was identified as an abundant oxidized protein in liver that decreases over time specifically in knockouts (Elchuri, Oberley et al. 2005). Similarly, in the *'toxic milk'* mouse mutant, Car3 protein is also greatly reduced in the liver; Car3 was shown to bind copper ions in addition to zinc (Grimes, Paynter et al. 1997). (Toxic milk protein is an ATP7B transporter that helps regulate copper levels in the body.) Thus Car3 seems to have physiological connections to both metallic ions and oxidative stress responses. While our study

has not demonstrated a role for Car3 in lipid metabolism, future characterization of *Car3 -/-* mice for roles in oxidative stress responses or for functions related to binding metal ions in cells may be more fruitful.

Conclusion

In conclusion, we attempted to determine the role of Car3 in lipid metabolism/homeostasis after we discovered Niclosamide decreased Car3 protein in the livers of HFD fed mice under similar conditions that improve fatty liver and insulin sensitivity/diabetic symptoms in mice (Tao, Zhang et al. 2014, Chowdhury, Turner et al. 2017). Since Niclosamide decreases liver triglycerides and improves insulin sensitivity, we hypothesized that perhaps Car3 had a functional role in promoting fatty acid synthesis or lipid accumulation. We determined using mouse genetics that Car3 is not required for *de novo* lipogenesis. Additionally, we found that Car3 knockout and wild type mice fed either a standard chow or a HFD are not statistically different in weight, body composition, metabolic rate, insulin sensitivity, serum lipids, or tissue triglycerides. While Car3 is an abundant protein in many key metabolic tissues, the physiological role of Car3 in these tissues still remains to be determined.

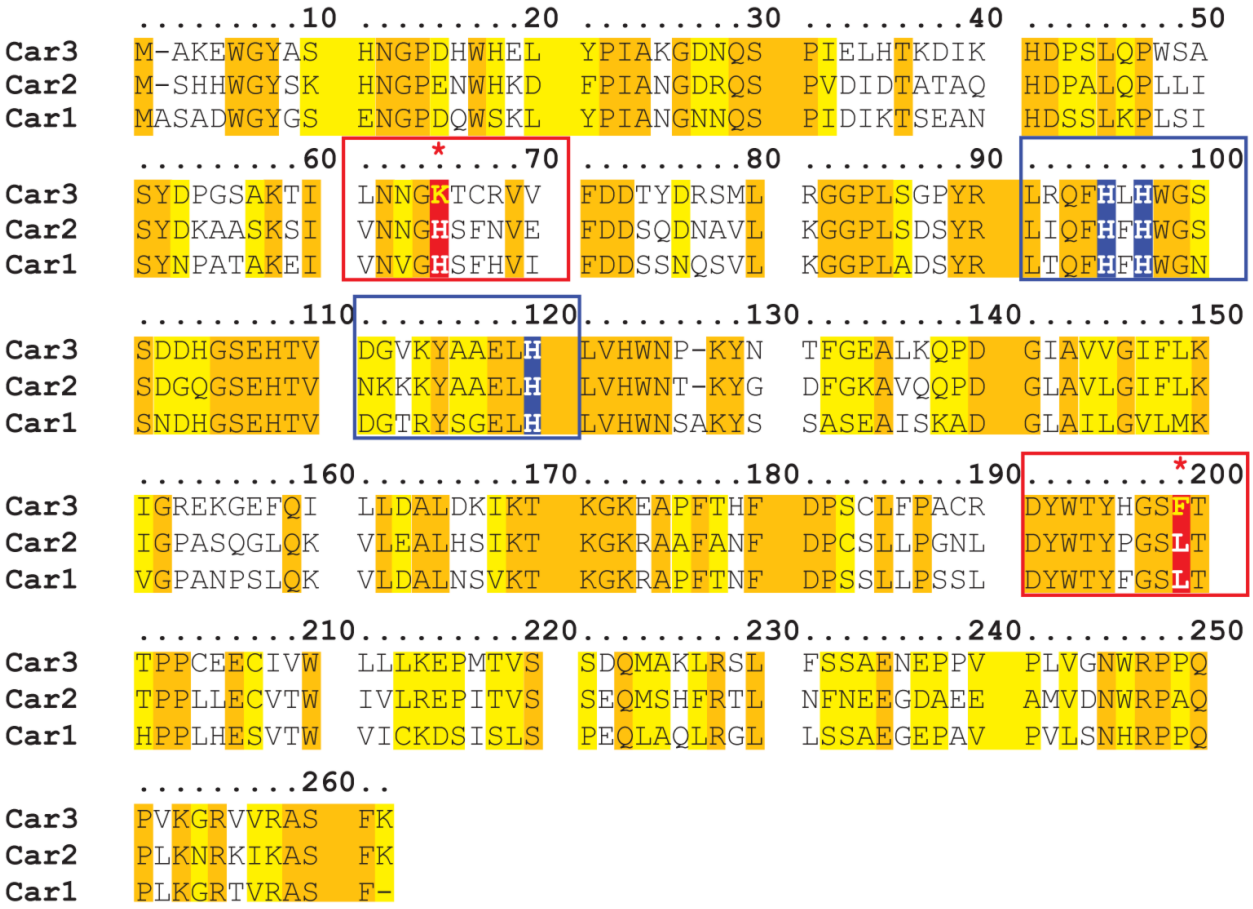

.....10 .....20 .....30 .....40 50
Car3 M-AKEWGYAS HNGPDHWHEL YPIAKGDNQS PIELHTKDIK HDPSLQPWSA
Car2 M-SHHWGYSK HNGPENWHKD FPIANGDRQS PVDIDTATAQ HDPALQPLLI
Car1 MASADWGYGS ENGPDQWSKL YPIANGNNQS PIDIKTSEAN HDSSLKPLSI
.....60 .....70 .....80 .....90 100
Car3 SYDPGSAKTI LNNGKTCRVV FDDTYDRSML RGGPLSGPYR LRQFHLHWGS
Car2 SYDKAASKSI VNNGHSFNVE FDDSQDNAVL KGGPLSDSYR LIQFHFHWGS
Car1 SYNPATAKEI VNVGHSFHVI FDDSSNQSVL KGGPLADSYR LTQFHFHWGN
.....110 .....120 .....130 .....140 150
Car3 SDDHGSEHTV DGVKYAAELH LVHWNP-KYN TFGEALKQPD GIAVVGIFLK
Car2 SDGQGSEHTV NKKKYAAELH LVHWNT-KYG DFGKAVQQPD GLAVLGIFLK
Car1 SNDHGSEHTV DGTRYSGELH LVHWNSAKYS SASEAISKAD GLAILGVLMK
.....160 .....170 .....180 .....190 200
Car3 IGREKGEFQI LLDALDKIKT KGKEAPFTHF DPSCLFPACR DYWTYHGSFT
Car2 IGPASQGLQK VLEALHSIKT KGKRAAFANF DPCSLLPGNL DYWTYPGSLT
Car1 VGPANPSLQK VLDALNSVKT KGKRAPFTNF DPSSLLPSSL DYWTYFGSLT
.....210 .....220 .....230 .....240 250
Car3 TPPCEECIVW LLLKEPMTVS SDQMAKLRSL FSSAENEPPV PLVGNWRPPQ
Car2 TPPLLECVTW IVLREPITVS SEQMSHFRTL NFNEEGDAEE AMVDNWRPAQ
Car1 HPPLHESVTW VICKDSISLS PEQLAQLRGL LSSAEGEPAV PVLSNHRPPQ
.....260..
Car3 PVKGRVVRAS FK
Car2 PLKNRKIKAS FK
Car1 PLKGRTVRAS F-

Figure 3.1. Amino acid sequence alignment of cytosolic carbonic anhydrases reveals key enzyme amino acids changes that affect activity. CA III (Car3) amino acid alignment with carbonic anhydrase family members Car1 and Car2. Car3 shares a 57%/56% identity and 74%/75% similarity with Car2 and Car1 respectively. Car3 has a 69% identity with either Car2 or Car1. Amino acids highlighted in yellow are identical between the three CAs. Amino acids highlighted in orange are shared between Car3 and either Car1 or Car2. The active site of all carbonic anhydrases contain a Zn^{2+} and three histidine residues (highlighted in blue) (Nishimori, Minakuchi et al. 2007). Two key amino acid differences (highlighted in red), at Lys64 and Phe198, are responsible for the substantially lower rate of regeneration of the active form for carbonic anhydrase 3 compared to the other two CAs (LoGrasso, Tu et al. 1993, Tu, Chen et al. 1994). A lysine at amino acid 64 results in a less effective proton shuttle, (An, Tu et al. 2002, Elder, Fisher et al. 2007) and Phe198 causes a steric hindrance at the active site (Duda, Tu et al. 2005).

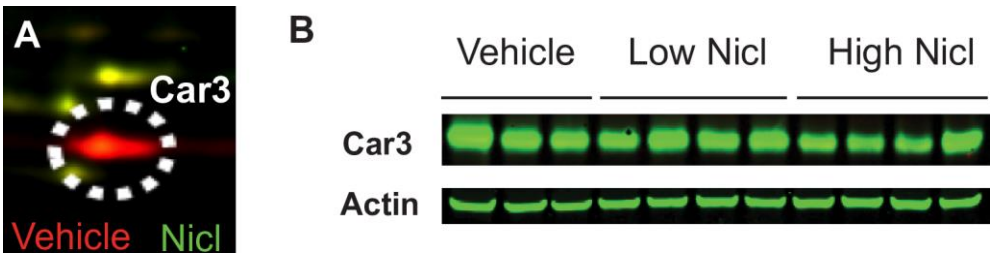


Figure 3.2. 2D-DIGE identifies Car3 as a protein with decreased expression in livers of high-fat diet fed mice treated with Niclosamide. (A) 2D-DIGE of liver lysates from 20 week-old diet-induced obesity (DIO) mice treated with 10mg/kg niclosamide (green) or vehicle (red) for 12 days. Car3 protein is highlighted in the white circle. (B) Independent validation by Western blot analysis of Car3 from DIO mice treated with vehicle, low niclosamide (5mg/kg), or high niclosamide (25mg/kg) for 12 days.

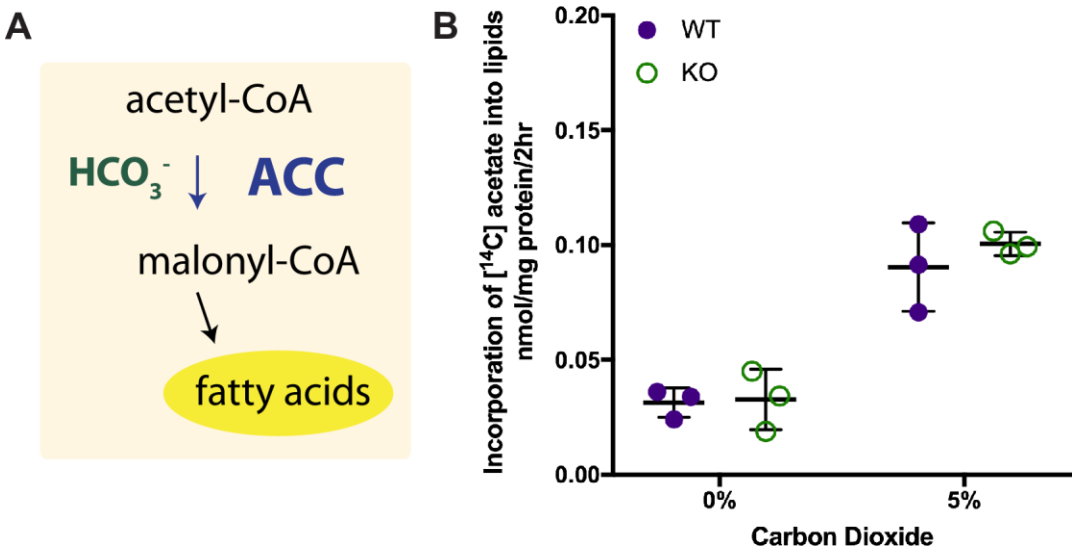


Figure 3.3. Car3 is not required for de novo lipogenesis. (A) The rate-limiting step in fatty acid synthesis requires bicarbonate for the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). (B) Wild type (WT) and *Car3 -/-* (KO) hepatocytes show no difference in *de novo* lipogenesis. Hepatocytes were labeled with [1,2-^{14}C] acetate in the presence and absence of carbon dioxide in bicarbonate free media. Data shown are from three experiments performed with six replicates.

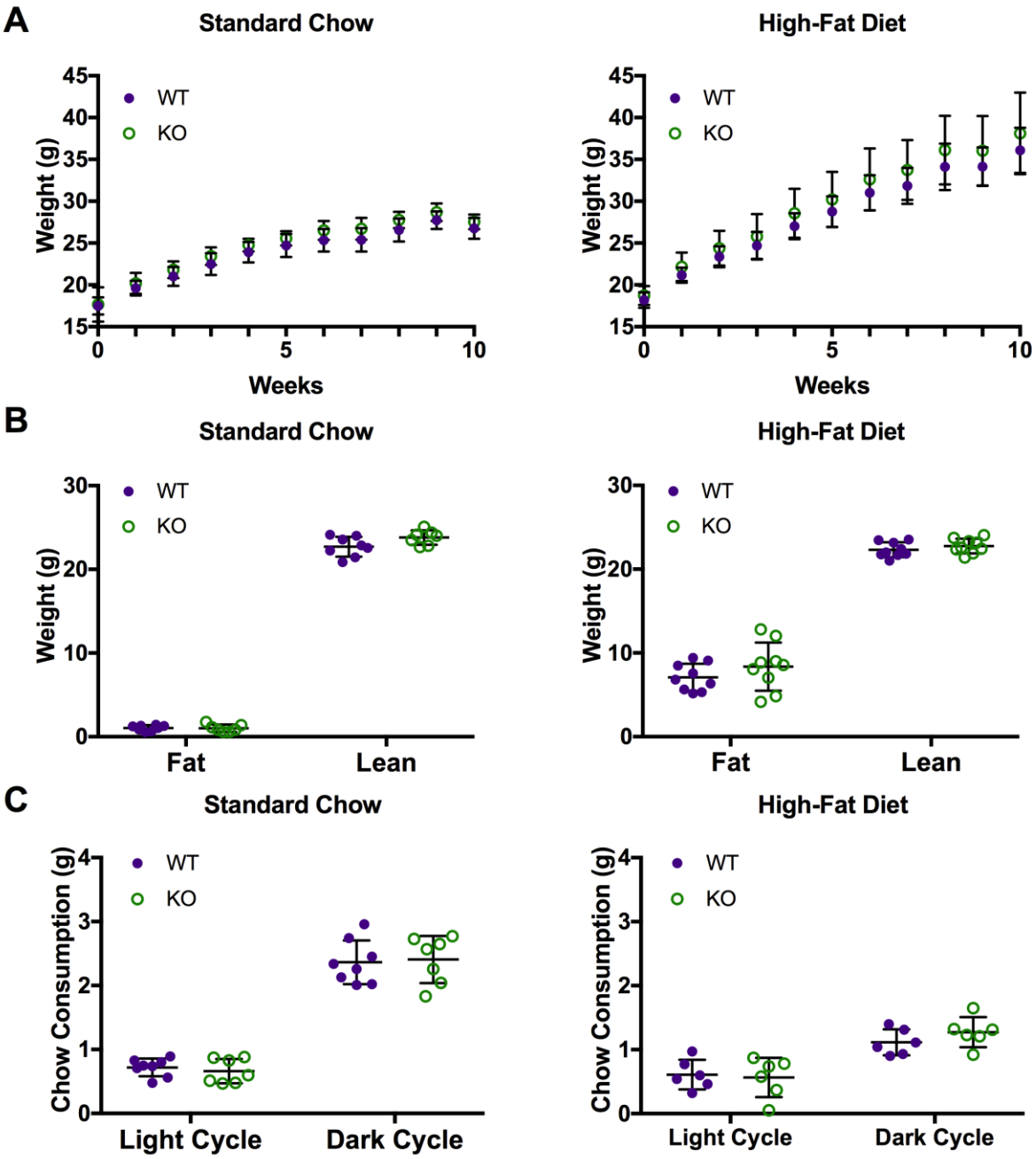

A
Standard Chow
High-Fat Diet
WT
KO
Weight (g)
Weeks
B
Standard Chow
High-Fat Diet
Fat
Lean
C
Standard Chow
High-Fat Diet
Chow Consumption (g)
Light Cycle
Dark Cycle

Figure 3.4. *Car3 -/-* mice fed high-fat diet showed no difference in weight gain, food consumption, or body composition. (A) Weight gain of wild type (WT) and *Car3 -/-* (KO) mice fed normal chow (n=7-8) or high-fat diet (n=9) starting at 5 weeks of age. (B) Body composition, fat and lean mass, of wild type and *Car3 -/-* mice measured at 11 weeks old by MRI. (C) Chow consumption of mice fed standard chow (n=7-8) or high-fat diet (n=6) during light and dark cycle measured at 13-14 weeks old.

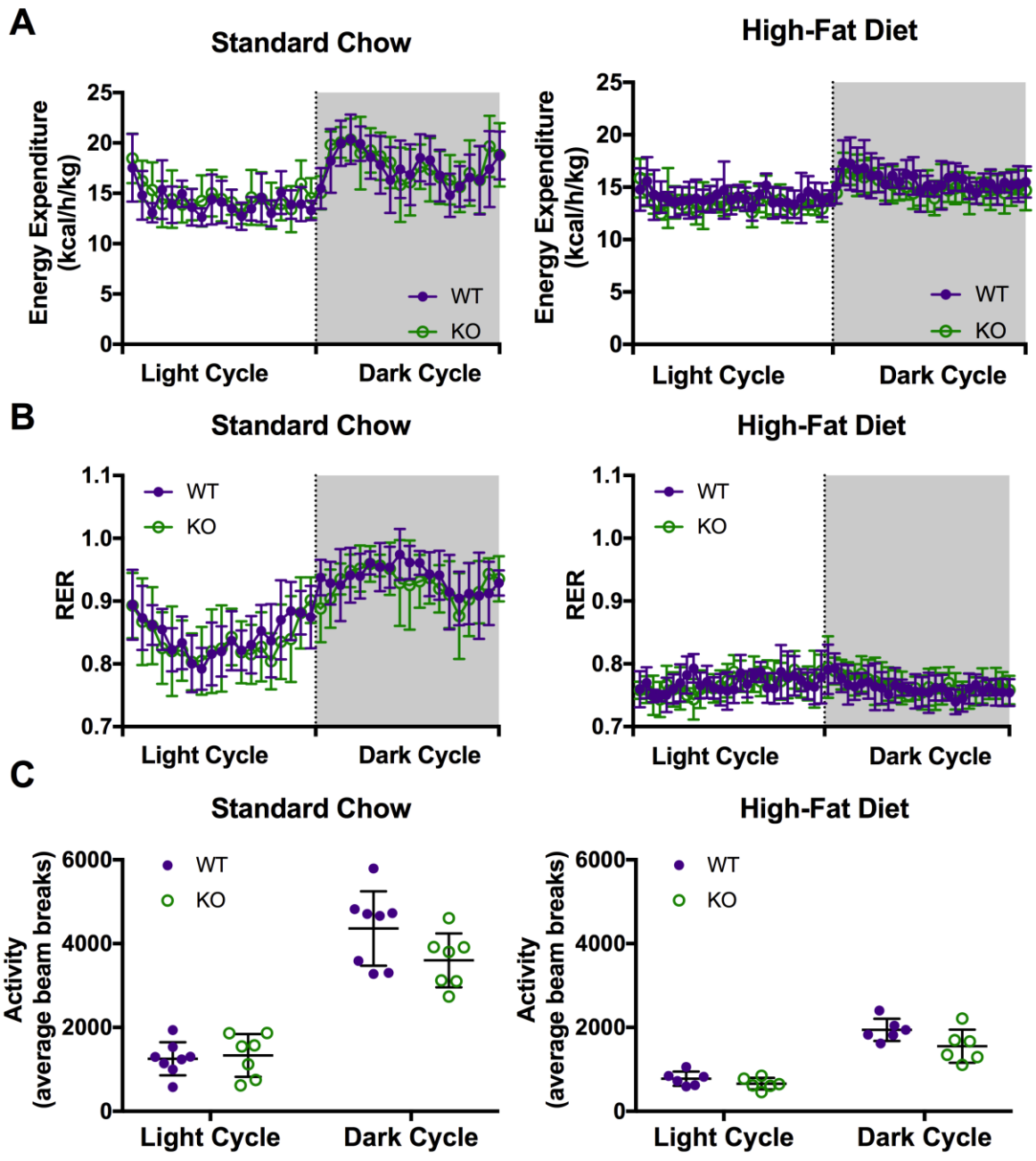

A
Standard Chow
High-Fat Diet
Energy Expenditure (kcal/h/kg)
25
20
15
10
5
0
WT
KO
Light Cycle
Dark Cycle
B
Standard Chow
High-Fat Diet
RER
1.1
1.0
0.9
0.8
0.7
C
Standard Chow
High-Fat Diet
Activity (average beam breaks)
6000
4000
2000
0

Figure 3.5. *Car3 -/-* mice show no changes in total energy expenditure, RER, or activity. Wild type (WT) and *Car3 -/-* (KO) energy expenditure (A), Respiratory Exchange Ratio (B), and activity (C) measured over a 24 hour period in 13-14-week-old mice on a standard chow (n=7-8) or high-fat diet (n=6). Standard chow fed to mice at weaning (3 weeks of age), mice fed high-fat diet were switched to HFD at 5 weeks of age.

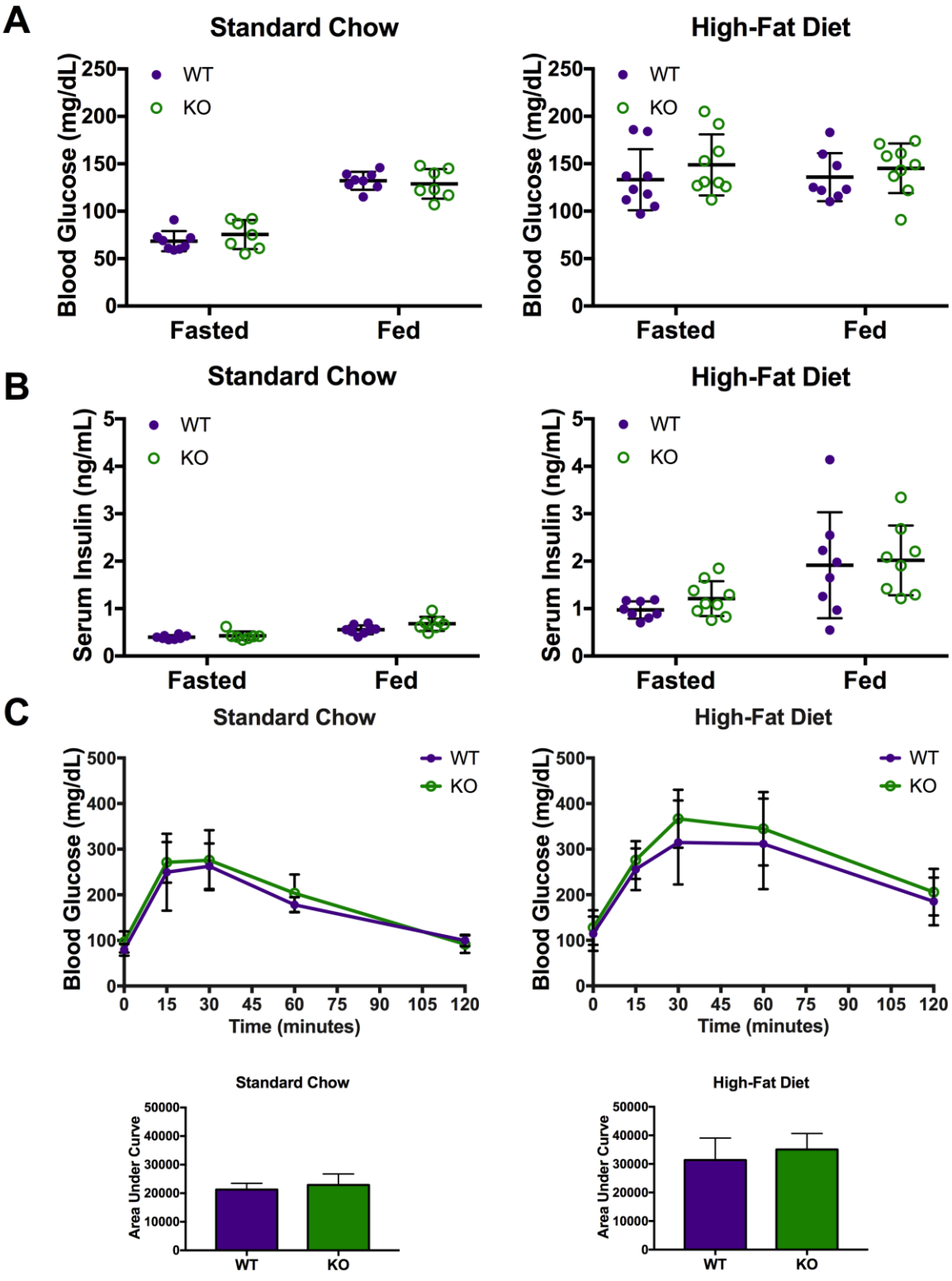

A
Standard Chow
High-Fat Diet
WT
KO
Blood Glucose (mg/dL)
0
50
100
150
200
250
Fasted
Fed
B
Standard Chow
High-Fat Diet
Serum Insulin (ng/mL)
0
1
2
3
4
5
C
Standard Chow
High-Fat Diet
Blood Glucose (mg/dL)
0
100
200
300
400
500
0 15 30 45 60 75 90 105 120
Time (minutes)
Standard Chow
High-Fat Diet
Area Under Curve
0
10000
20000
30000
40000
50000
WT
KO

Figure 3.6. *Car3 -/-* does not affect high-fat diet-induced insulin-resistance. Fasting and fed blood glucose (A) and serum insulin (B) from wild type (WT) and *Car3 -/-* (KO) mice fed standard chow (n=7-8) or high-fat diet (n=8-9) for 10 weeks. (C) Oral glucose tolerance test (with area under the curve below) performed on standard chow (n=7-8) and high-fat diet (n=4-5) mice after 11 weeks on the respective diet regime.

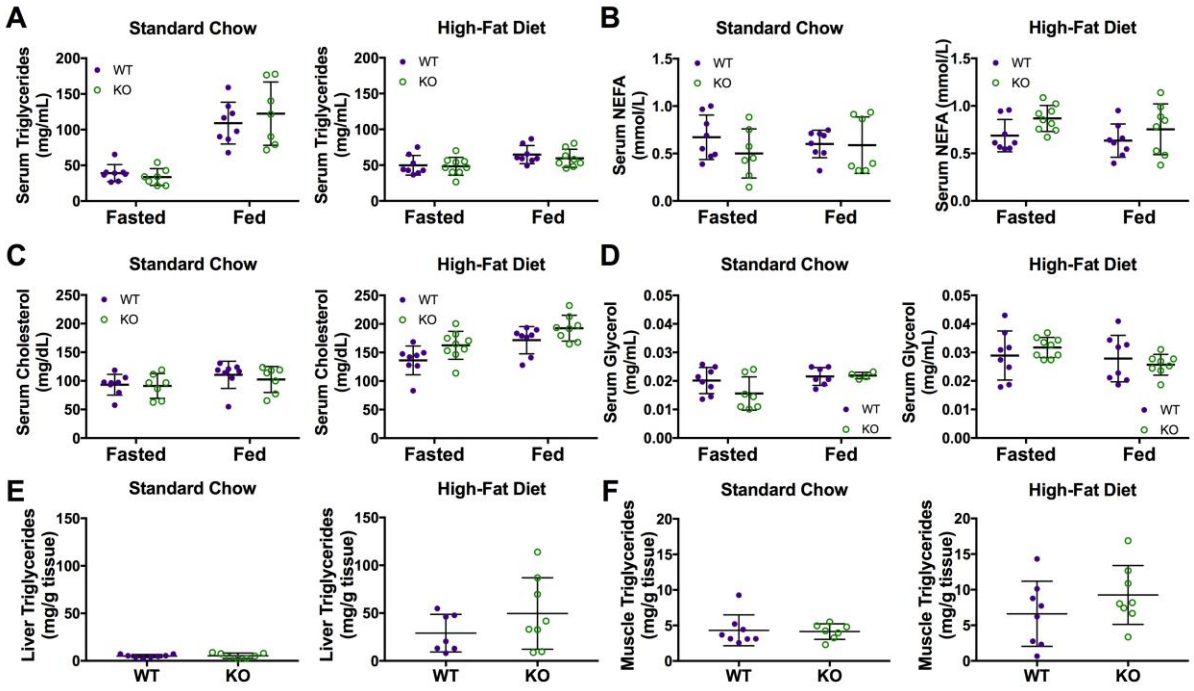


Figure 3.7. *Car3 -/-* mice show no difference in serum and tissue lipids and lipid substrates. Fasting and fed serum triglycerides (A), non-esterfied fatty acids (B), cholesterol (C), and glycerol (D) of wild type (WT) and *Car3 -/-* (KO) mice fed standard chow (n=7-8) or high-fat diet (n=8-9) for 10 weeks. Liver (E) and muscle (F) triglyceride content in mice fed standard chow (n=7-8) or high-fat diet (n=8) for 13 weeks.

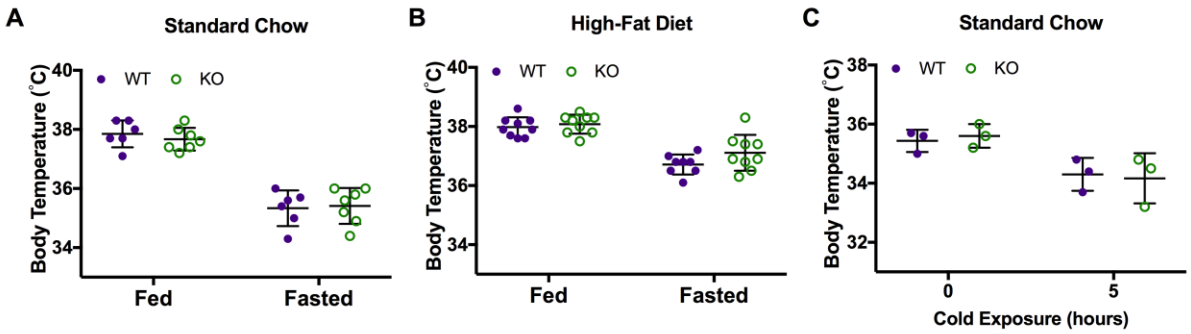


Figure 3.8. ***Car3 -/-*** **mice have normal thermoregulation**. Wild type (WT) and knockout (KO) body temperatures of adult mice aged 8-15 weeks at different states of thermoregulation. Fasted state is an overnight fast for 15-18 hours. (A) Females fed standard chow (n=6-7). (B) Males fed HFD for 10 weeks, starting at age 5 weeks (n=8-9) (C) Females were fasted overnight and subjected to 4°C for 5 hours (n=3).

CHAPTER 4 – CONCLUSIONS & FUTURE DIRECTIONS FOR CA III STUDIES

Conclusions

Role of CA III still unknown but future areas for exploration are more clear

This dissertation investigated the function of CA III in lipid metabolism. We unfortunately did not find a genetic requirement for CA III in lipid synthesis. However, we made some progress in understanding mechanistic pathways CA III is not required for function. We observed that CA III protein expression decreases in livers of mice treated with niclosamide, a drug that improves insulin sensitivity and reduces liver triglycerides in HFD-induced fatty liver mice (Renner, Walker et al. 2017). Elevated CA III in untreated fatty livers was consistent with prior CA III expression data that showed CA III expression was high in nutrition rich states (mentioned in Chapter 2) and also indicated that CA III expression was correlated with the amount of lipid in liver. This implied a role for CA III in lipid metabolism, either through promoting *de novo* lipogenesis and/or lipid storage, or regulating lipid breakdown.

We subsequently found that CA III is not required for *de novo* synthesis and a high-fat diet is not enough of a metabolic stressor to induce gross differences in lipid metabolism between Car3 WT and KO mice (Renner, Walker et al. 2017). Since we did not directly address lipid storage or lipolysis, more research is required to address CA III functionality in the progression of fatty liver disease and lipid metabolism.

Potential confounding variables

Why we did not find significant differences in Car3 WT and KO mice

We found no significant differences between Car3 WT and KO mice in any of the assays we performed: glucose tolerance, weight gain on different diets, liver triglycerides, metabolic chambers, or tissue weights (Renner, Walker et al. 2017). We cannot rule out the possibility that CA III is involved in lipid metabolism, rather it is possible the effects were too small to be reliably quantified or we were not directly testing the specific metabolic pathway affected by CA III. While we controlled for variables as much as possible, there are many confounding variables that could explain why we saw no significant differences between Car3 WT and KO mice: timing, temperature, lack of appropriate stressor, or genetic compensation.

In humans and other animals, biological clocks, or circadian rhythms, anticipate and assist in the timing of important physiological functions like sleeping and eating through the regulation of hormones and metabolic pathways (Maury, Ramsey et al. 2010). To avoid disturbing the circadian rhythms, access to light is well regulated in mouse facilities with 12 hours of daylight and 12 hours of darkness. Even though we controlled for blood and tissue collection time, it is possible we missed differences in metabolites due to the timing of peak circadian rhythm hormone/lipid/metabolite production (Kalsbeek and Strubbe 1998) (Maury, Ramsey et al. 2010) (Ferrell and Chiang 2015). Similarly, it is possible we missed minor differences in fasted metabolites by not optimizing the fasting duration. Metabolites and hormones are dynamically regulated and can significantly change depending on fasting duration (Geisler, Hepler et al. 2016). It is possible that there was a minor difference in glucose, fatty acids, or other metabolites tested in the blood that was unable to be seen during our experimental window.

The Car3 WT and KO mice were housed at room temperature (~20-23°C). While a commonly accepted practice, mice raised and studied below thermoneutral condition (~28-30°C), can have altered metabolic phenotypes: impaired glucose homeostasis, higher oxygen consumption, and increased food consumption (Dudele, Rasmussen et al. 2015). Specifically, mice have a shivering mechanism independent of uncoupling, lipolysis, and fatty acid oxidation that can mask changes in these pathways when tested below thermoneutral conditions. For example, UCP1 KO mice were protected from obesity in initial studies done at room temperature due to the secondary shivering mechanism, however when studied in thermoneutral conditions, where the secondary mechanism would not be necessary the UCP1 KO mice gained more weight than UCP1 WT mice (Feldmann, Golozoubova et al. 2009). It is possible that differences in weight gain or lipid metabolism in the Car3 KO mice were masked by the secondary shivering mechanism.

While duplicate mechanisms can compensate for alterations in metabolism, like the UCP1 KO mice, isozymes of the α-CA family can also potentially compensate for loss of CA III in mice. For example, studies previously mentioned in Chapter 1 with CA VA and VB single and double KO mice showed that CA VA could compensate for the loss of CA VB (Shah, Rubbelke et al. 2013). Additionally, humans deficient in CA I are able to compensate for this loss, as they have no disease phenotypes (Sly and Hu 1995). It is possible that there are unknown compensatory mechanisms in mice for CA III depletion that we were unaware of. Co-depletion of other similar enzymes with CA III may give us stronger disease phenotypes. A possible compensatory enzyme could be CA VII, as CA III and CA VII are the only CA isozymes that can be S-glutathionylated and they have a similar tissue distribution.

These studies were conducted using common experimental procedures in order to determine a disease phenotype of CA III and to help elucidate a role for CA III in metabolism. It is possible that the role of CA III in metabolism is subtle and therefore requires a more detailed exploration of phenotypes. Future studies using CA III KO mice should consider a more detailed procedure with stricter phenotypic analysis and consider focusing more strongly on the following experimental procedures: careful and precise timing of blood draws and tissue extractions, stricter control of temperature, and potentially utilizing double knockout mice in order to test for compensatory mechanisms of CA III with other carbonic anhydrases.

Future directions

CA III expression and regulation in fatty liver disease

CA III expression data previously indicated a decrease in CA III protein and mRNA when fatty liver is induced with particular mouse models: alcohol, methionine deficient diets, and carbon tetrachloride (Aroor, Roy et al. 2012) (Ronchi, Conde et al. 2004, Kharbanda, Vigneswara et al. 2009, Yamada, Satoh et al. 2013, Carter, Vigneswara et al. 2015) (Sanllorenti, Rosenfeld et al. 2001, Henkel, Roderfeld et al. 2006, Wong, Fan et al. 2011, Eakins, Walsh et al. 2015). These results implied that CA III was protecting the liver from oxidative damage and the decrease in CA III contributed to the diseased livers. However, our data suggested that CA III was elevated in HFD-induced fatty livers and perhaps promoted the accumulation of fat in livers (Renner, Walker et al. 2017). As discussed earlier in Chapter 2, fatty liver disease progression is thought to have three stages: fat accumulation, inflammation and oxidative damage, and lastly fibrosis and death of the liver. The fatty liver disease model we used, HFD-induced, contributes to the first stage of fat accumulation whereas alcohol, methionine deficient diets, and carbon tetrachloride also directly contribute to the later stage of inflammation and oxidative damage

(Anstee and Goldin 2006, Lau, Zhang et al. 2017). HFD indirectly induces oxidative damage as it causes obesity and dyslipidemia, which contribute to the induction of oxidative stress (Hutcheson and Rocic 2012). Enzymes critical for dealing with reactive oxygen species (ROS) are down regulated during oxidative damage (Hutcheson and Rocic 2012), and the subsequent increased ROS production damages phospholipid membranes of organelles, increases lipid peroxidation, and releases pro-inflammatory cytokines (Anstee and Goldin 2006, Le Lay, Simard et al. 2014). While we do not fully understand the role of CA III in fatty liver disease, we speculate that CA III either promotes a lipid storage/accumulation pathway that is down-regulated with increased ROS, or CA III protects the liver against oxidative damage under normal conditions and increased ROS decreased the expression of CA III.

Measuring CA III protein modification by oxidative stress and metabolism

A majority of our research focused on lipid metabolism, however we wanted to address the possibility that CA III might be protecting livers from oxidative damage. We found no difference in oxidation of proteins in livers of Car3 WT and KO mice under diverse metabolic conditions: fed different diets and under starved conditions (Figure 4.1). Our result suggested that CA III is not required for oxidative stress response, although lipid peroxidation products and other indicators of oxidative damage were not tested.

While Car3 KO mice showed no differences in response to oxidative damage, our experimentation was not exhaustive. The understanding of CA III and its role in oxidative damage is very basic; it can be S-glutathionylated and its expression is decreased during oxidative stress. Instead of characterizing the phenotypes of CA III mice, further experiments should focus why CA III expression decreases during oxidation. One possibility is that CA III is not as stable when oxidized. To test if CA III protein stability is affected by oxidative damage

through S-glutathionylation, we could perform ^{35}S-radiolabeled amino acid pulse-chase analysis under normal and oxidized conditions in cell lines that express CA III, like differentiated 3T3-L1 or sol8 (Zhou 2004). By comparing the half-life of CA III under oxidized and non-oxidized conditions, we should be able to determine if protein turnover is directly affected by oxidation. Additionally, we can use isoelectric focusing to visualize differences in S-glutathionylation during protein-turnover and oxidative damage (Chai, Jung et al. 1991). We could also experiment with inhibiting or reversing S-glutathionylation, by incubating cells with and without an abundance of glutathione (which reverses S-glutathionylation of CA III). This could help us determine if this post-translational modification effects CA III protein stabilization (Chai, Jung et al. 1991).

Determining if CA III negatively regulates lipolysis

From our finding that CA III protein is decreased in livers treated with niclosamide in mice, we speculated that CA III could be promoting oxidative damage response, or altering lipid metabolism either by promoting *de novo* lipogenesis and fat storage, or inhibiting lipolysis. We anticipated that deficiencies in lipid metabolism would have been detectable in our varied assays, yet we saw no difference in Car3 WT and KO mice (Renner, Walker et al. 2017). The assays used did not directly examine the hypothesis that CA III inhibits lipolysis, the degradation of triglycerides into fatty acids and glycerol.

To test if CA III is involved in the signaling pathways for lipolysis, we could induce lipolysis in Car3 WT and KO primary cells, tissues, or *in vivo* with isoproterenol or another β-adrenergic agonist and measure the products of TG breakdown (fatty acids and glycerol) (Rutkowski, Stern et al. 2015). Elevated products of lipolysis in Car3 KO mice would confirm a

role for CA III in regulating lipolysis. If there were a difference in lipolysis, further experimentation would be required to determine the regulatory mechanism.

Understanding role of CA III in lipid droplets

Lipolysis occurs on the membranes of lipid droplets (LDs) in cells. Lipid droplets are organelles composed of neutral fats—triglycerides and sterol esters—incased in a phospholipid monolayer (Walther and Farese 2012). Proteins associated with LDs can alter storage capacity and stability of LDs (Barneda and Christian 2017) (Walther and Farese 2012). Many lipid droplet-associated proteins are involved in regulating lipolysis, the breakdown of triglycerides into fatty acids and glycerol (Rutkowski, Stern et al. 2015).

CA III protein has been found in many different purified lipid droplet isolations from hepatocytes and adipocytes of rodents (Kanshin, Wang et al. 2009) (Ding, Wu et al. 2012) (Crunk, Monks et al. 2013, Khan, Wollaston-Hayden et al. 2015). While it is possible that CA III is so abundant in the cytosol that it contaminates the LD layers in isolations, the association with LDs seems plausible. As a negative control, lipid droplets fractions were probed for many abundant proteins in the cytosol, cell membranes, and mitochondria. The abundant cytosolic proteins, GAPDH and actin, were not found in the LD fractions (Kanshin, Wang et al. 2009) (Ding, Wu et al. 2012) (Khan, Wollaston-Hayden et al. 2015). Determining how CA III is associated with lipid droplets would verify the studies that found CA III in the lipid droplet proteome.

LD associated proteins typically interact with the monolayer membrane in a variety of methods (Walther 2012); CA III likely associates with LDs binding to lipids or proteins that bind to the membrane. CA III has the potential to bind to lipids; the catalytic mechanism of carbonic anhydrase includes a stage in which a carboxyl group is bound to the active site. Since CA III

has alterations in amino acids near its active site compared to other cytosolic α-CAs, it is possible that instead of catalyzing the carboxylation of water CA III binds to the carboxyl groups of fatty acids, especially small chain fatty acids (Figure 4.2) (Supuran 2008). Lipid interactions with CA III could help explain how CA III associates with LDs, however CA III could also simply bind a protein already bound to the LD membrane.

To determine if CA III binds to fatty acids or other lipids, we could affinity purify CA III from adipose cell or tissue lysates, extract bound lipids, and send to liquid chromatography mass spectroscopy following similar methodologies as Li *et al.* that found lipids and metabolites which bind to proteins (Li, Gianoulis et al. 2010). By adjusting the buffer utilized, protein-protein interactions could also be measured using immunoprecipitation. It would be interesting to find any protein-protein interactions, not only to potentially determine how CA III associates with LDs but interactions could identify pathways that could guide future experiments to determine CA III functionality.

Preliminary data- Car3 KO mice have increased water intake during night starvation

In our published data, we were unable to find significant differences between Car3 WT and KO mice fuel utilization under fed conditions. However, an experiment performed during the writing of this document showed that fasted Car3 KO mice drank nearly twice as much water overnight compared to Car3 WT (Figure 4.3). This change was only seen during dark cycle of the fasted state, and there were no genotype differences in body weight, RER, energy expenditure, or movement.

We made efforts to reproduce the drinking phenomenon without using the metabolic chamber, however this method was too crude, and measurements were inaccurate due to leakage of the generic animal facility water bottles. Additionally, the writing of this document and

wrapping up the CA III project prevented further follow up. While this experiment was not replicated, we thought its inclusion in this dissertation was important to illustrate a striking phenotype in the Car3 WT and KO mice that has never before been detected.

The increased water intake in Car3 KO mice is puzzling, as it is not intuitive what drinking behavior has to do with CA III. Assuming the data replicate, there are three plausible hypotheses that could explain the increase in water consumption: 1) water is elevated to excrete a metabolite that accumulates in Car3 KO mice, 2) thirst is somehow regulated by CA III, 3) starvation and introduction to a new environment elevates stress hormones that affect drinking differentially in Car3 KO mice.

Water is required to excrete wastes in urine. If the concentration of waste metabolites is greater than normal, more water is required to flush the kidneys to remove these compounds (Choi, Park et al. 2015). The CA III deficiency could be triggering an accumulation of waste, which could be a number of different compounds: sugars, urea, salts, or toxins (Bouatra, Aziat et al. 2013). Narrowing down potential pathways would be purely speculative without knowing if there is an increase in urine production. To determine if Car3 KO mice are drinking more because they are flushing a waste compounds in the urine, urine production of starved Car3 WT and KO mice should be measured and collected urine sent for metabolomic analysis (Bouatra, Aziat et al. 2013).

Water intake is mostly regulated though osmotic pressure and sodium-sensitive receptors in the brain (Fuller 1984) (Popkin, D'Anci et al. 2010). In response to osmotic pressure, the thirst hormone, arginine vasopressin is elevated and induces thirst (Popkin, D'Anci et al. 2010). Some hormones not typically involved in regulating water intake can also increase water consumption; elevated cortisol and epinephrine, a glucocorticoid and catecholamine respectively, have been

shown to increase water intake (Katovich and Fregly 1978, Fuller 1984, Johnson, Beltz et al. 2015). It is possible that Car3 KO mice have elevated thirst-inducing hormones while fasting, which causes these mice to consume more water.

In addition to regulating water intake, vasopressin, insulin, glucocorticoids, and catecholamines all respond to stressful conditions (Ranabir and Reetu 2011). The Car3 WT and KO mice were subjected to two potential stressors, starvation and a new environment, that could have induced a more potent stress response in Car3 KO mice than the WT mice. To determine if Car3 KO mice are drinking more water through alterations in the water intake pathway or as a response to stress would require further testing. Basal and fasted hormones associated with water intake, arginine vasopressin, glucocorticoids, and catecholamines should be tested in blood and tissues from the Car3 WT and KO mice. This assay would determine if there are any differences in hormonal regulation of water intake or stress response and help define further exploration of CA III in either water intake pathways or responses to acute stress.

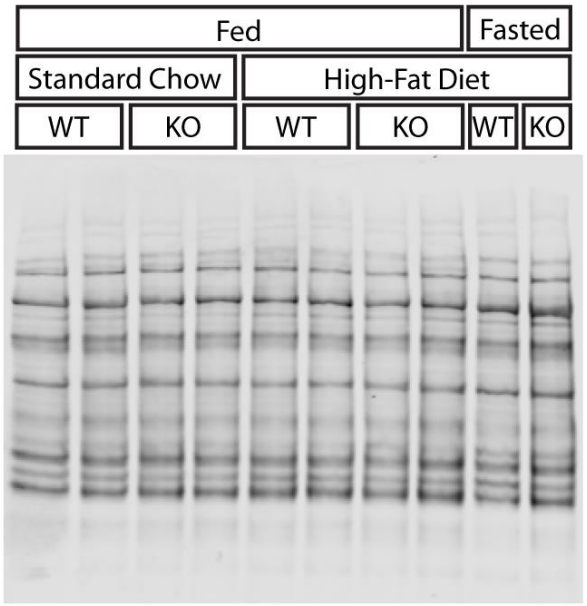


Figure 4.1. CA III has no effect on oxidation of proteins in livers of mice. Oxidized proteins from liver lysates of fed and fasted Car3 WT and KO mice. Liver lysates were DNP-derivatized, which marks the carbonyl groups of proteins and is an indicator of oxidative protein modification. (Abcam Oxidized Protein Western (ab178020)).

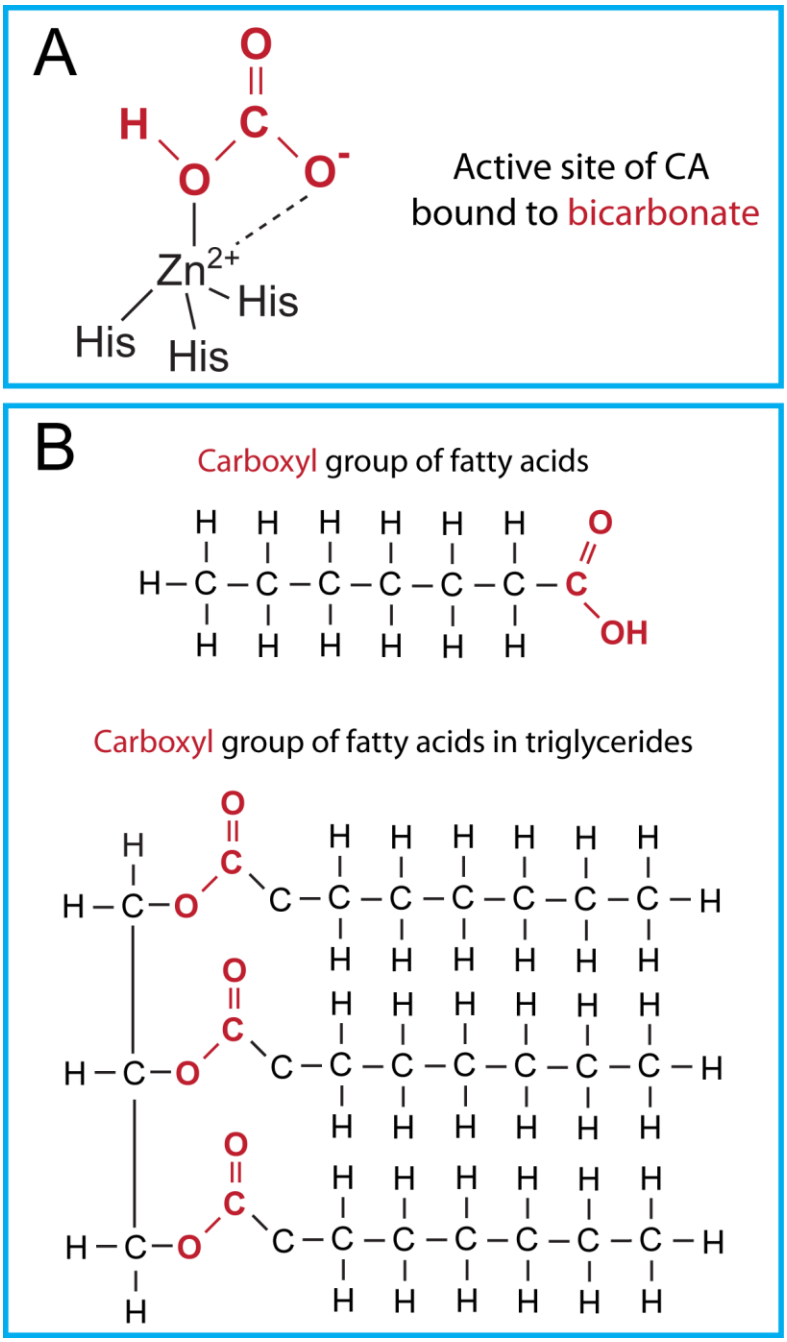


Figure 4.2. Potential binding of CA with carboxyl groups of lipids. (A) CAs bind bicarbonate which contains a carboxyl group during catalysis. (B) Single fatty acids and fatty acids bound to glyceride backbones both have carboxyl groups that could bind to the active site of CA.

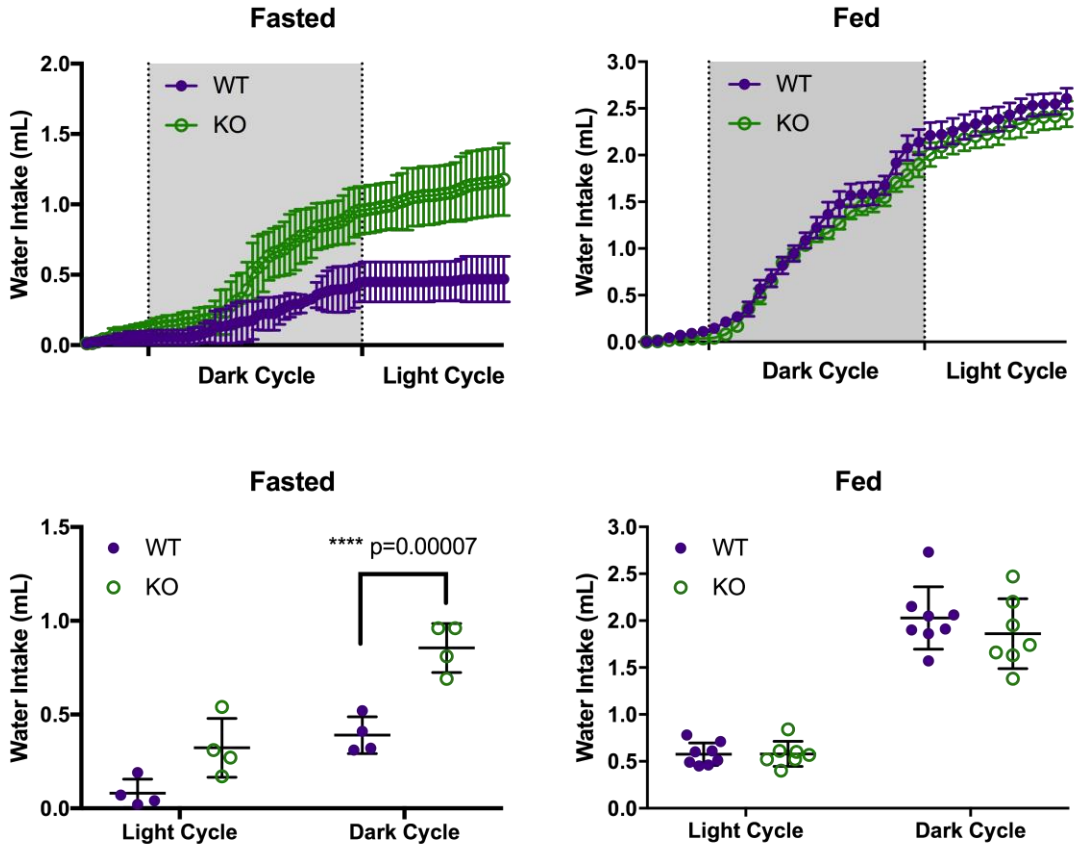


Figure 4.3. Car3 -/- mice drink more water than WT mice when fasting. Fasted female mice (n=4) were starved at 3pm. Fed male mice (n=7-8) had *ad libitum* access to standard chow and water.

REFERENCES

Aggarwal, M., C. D. Boone, B. Kondeti and R. McKenna (2013). "Structural annotation of human carbonic anhydrases." J Enzyme Inhib Med Chem **28**(2): 267-277.

Almind, K. and C. R. Kahn (2004). "Genetic determinants of energy expenditure and insulin resistance in diet-induced obesity in mice." Diabetes **53**(12): 3274-3285.

Alver, A., F. Ucar, E. E. Keha, E. Kalay and E. Ovali (2004). "Effects of leptin and insulin on CA III expression in rat adipose tissue." J Enzyme Inhib Med Chem **19**(3): 279-281.

An, H., C. Tu, K. Ren, P. J. Laipis and D. N. Silverman (2002). "Proton transfer within the active-site cavity of carbonic anhydrase III." Biochim Biophys Acta **1599**(1-2): 21-27.

Andrikopoulos, S., C. M. Massa, K. Aston-Mourney, A. Funkat, B. C. Fam, R. L. Hull, S. E. Kahn and J. Proietto (2005). "Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high fat diet." J Endocrinol **187**(1): 45-53.

Anstee, Q. M. and R. D. Goldin (2006). "Mouse models in non-alcoholic fatty liver disease and steatohepatitis research." Int J Exp Pathol **87**(1): 1-16.

Arias-Hidalgo, M., J. Hegermann, G. Tsiavaliaris, F. Carta, C. T. Supuran, G. Gros and V. Endeward (2016). "CO2 and HCO3- Permeability of the Rat Liver Mitochondrial Membrane." Cell Physiol Biochem **39**(5): 2014-2024.

Aroor, A. R., L. J. Roy, R. J. Restrepo, B. P. Mooney and S. D. Shukla (2012). "A proteomic analysis of liver after ethanol binge in chronically ethanol treated rats." Proteome Sci **10**(1): 29.

Aspatwar, A., M. E. Tolvanen, C. Ortutay and S. Parkkila (2010). "Carbonic anhydrase related protein VIII and its role in neurodegeneration and cancer." Curr Pharm Des **16**(29): 3264-3276.

Aspatwar, A., M. E. Tolvanen, C. Ortutay and S. Parkkila (2014). "Carbonic anhydrase related proteins: molecular biology and evolution." Subcell Biochem **75**: 135-156.

Aspatwar, A., M. E. Tolvanen and S. Parkkila (2010). "Phylogeny and expression of carbonic anhydrase-related proteins." BMC Mol Biol **11**: 25.

Ayala, J. E., V. T. Samuel, G. J. Morton, S. Obici, C. M. Croniger, G. I. Shulman, D. H. Wasserman, O. P. McGuinness and N. I. H. M. M. P. C. Consortium (2010). "Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice." Dis Model Mech **3**(9-10): 525-534.

Badger, M. R. and G. D. Price (1994). "The Role of Carbonic-Anhydrase in Photosynthesis." Annual Review of Plant Physiology and Plant Molecular Biology **45**: 369-392.

Barneda, D. and M. Christian (2017). "Lipid droplet growth: regulation of a dynamic organelle." Curr Opin Cell Biol **47**: 9-15.

Bartelt, A., O. T. Bruns, R. Reimer, H. Hohenberg, H. Ittrich, K. Peldschus, M. G. Kaul, U. I. Tromsdorf, H. Weller, C. Waurisch, A. Eychmuller, P. L. Gordts, F. Rinninger, K. Bruegelmann, B. Freund, P. Nielsen, M. Merkel and J. Heeren (2011). "Brown adipose tissue activity controls triglyceride clearance." Nat Med **17**(2): 200-205.

Bauer, M., A. C. Hamm, M. Bonaus, A. Jacob, J. Jaekel, H. Schorle, M. J. Pankratz and J. D. Katzenberger (2004). "Starvation response in mouse liver shows strong correlation with life-span-prolonging processes." Physiological genomics **17**: 230-244.

Benard, O., J. Lim, P. Apontes, X. Jing, R. H. Angeletti and Y. Chi (2016). "Impact of high-fat diet on the proteome of mouse liver." J Nutr Biochem **31**: 10-19.

Benej, M., S. Pastorekova and J. Pastorek (2014). "Carbonic anhydrase IX: regulation and role in cancer." Subcell Biochem **75**: 199-219.

Benjamini, Y., A. M. Krieger and D. Yekutieli (2006). "Adaptive linear step-up procedures that control the false discovery rate." Biometrika **93**(3): 491-507.

Berglund, E. D., C. Y. Li, G. Poffenberger, J. E. Ayala, P. T. Fueger, S. E. Willis, M. M. Jewell, A. C. Powers and D. H. Wasserman (2008). "Glucose metabolism in vivo in four commonly used inbred mouse strains." Diabetes **57**(7): 1790-1799.

Biddinger, S. B., K. Almind, M. Miyazaki, E. Kokkotou, J. M. Ntambi and C. R. Kahn (2005). "Effects of diet and genetic background on sterol regulatory element-binding protein-1c, stearoyl-CoA desaturase 1, and the development of the metabolic syndrome." Diabetes **54**(5): 1314-1323.

Bondia-Pons, I., N. Boque, L. Paternain, E. Santamaria, J. Fernandez, J. Campion, F. Milagro, F. Corrales and J. A. Martinez (2011). "Liver proteome changes induced by a short-term high-fat sucrose diet in wistar rats." J Nutrigenet Nutrigenomics **4**(6): 344-353.

Bootorabi, F. H., J; Smith, E; Haapasalo, H; Parkkila, S (2011). "Carbonic anhydrase VII-a potential prognostic marker in gliomas." Health **3**(1): 6-12.

Bouatra, S., F. Aziat, R. Mandal, A. C. Guo, M. R. Wilson, C. Knox, T. C. Bjorndahl, R. Krishnamurthy, F. Saleem, P. Liu, Z. T. Dame, J. Poelzer, J. Huynh, F. S. Yallou, N. Psychogios, E. Dong, R. Bogumil, C. Roehring and D. S. Wishart (2013). "The human urine metabolome." PLoS One **8**(9): e73076.

Brosnan, J. T. and M. E. Brosnan (2006). "The sulfur-containing amino acids: an overview." J Nutr **136**(6 Suppl): 1636S-1640S.

Caballero, V. J., J. R. Mendieta, A. M. Giudici, A. C. Crupkin, C. G. Barbeito, V. P. Ronchi, A. N. Chisari and R. D. Conde (2011). "Alternation between dietary protein depletion and normal feeding cause liver damage in mouse." J Physiol Biochem **67**(1): 43-52.

Cabiscol, E. and R. L. Levine (1995). "Carbonic anhydrase III. Oxidative modification in vivo and loss of phosphatase activity during aging." J Biol Chem **270**(24): 14742-14747.

Carter, W. G., V. Vigneswara, A. Newlaczyl, D. Wayne, B. Ahmed, S. Saddington, C. Brewer, N. Raut, H. K. Gerdes, A. M. Erdozain, D. Tooth, E. L. Bolt, N. A. Osna, D. J. Tuma and K. K. Kharbanda (2015). "Isoaspartate, carbamoyl phosphate synthase-1, and carbonic anhydrase-III as biomarkers of liver injury." Biochem Biophys Res Commun **458**(3): 626-631.

Chai, Y. C., C. H. Jung, C. K. Lii, S. S. Ashraf, S. Hendrich, B. Wolf, H. Sies and J. A. Thomas (1991). "Identification of an abundant S-thiolated rat liver protein as carbonic anhydrase III; characterization of S-thiolation and dethiolation reactions." Arch Biochem Biophys **284**(2): 270-278.

Champy, M. F., M. Selloum, L. Piard, V. Zeitler, C. Caradec, P. Chambon and J. Auwerx (2004). "Mouse functional genomics requires standardization of mouse handling and housing conditions." Mamm Genome **15**(10): 768-783.

Champy, M. F., M. Selloum, V. Zeitler, C. Caradec, B. Jung, S. Rousseau, L. Pouilly, T. Sorg and J. Auwerx (2008). "Genetic background determines metabolic phenotypes in the mouse." Mamm Genome **19**(5): 318-331.

Chegwidden, W. R. and N. D. Carter (2000). "Introduction to the carbonic anhydrases." EXS(90): 14-28.

Chegwidden, W. R., S. J. Dodgson and I. M. Spencer (2000). "The roles of carbonic anhydrase in metabolism, cell growth and cancer in animals." EXS(90): 343-363.

Choi, H. Y., H. C. Park and S. K. Ha (2015). "High Water Intake and Progression of Chronic Kidney Diseases." Electrolyte Blood Press **13**(2): 46-51.

Choi, J. H., A. S. Banks, J. L. Estall, S. Kajimura, P. Boström, D. Laznik, J. L. Ruas, M. J. Chalmers, T. M. Kamenecka, M. Blüher, P. R. Griffin and B. M. Spiegelman (2010). "Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5." Nature **466**: 451-456.

Choi, J. H., A. S. Banks, T. M. Kamenecka, S. A. Busby, M. J. Chalmers, N. Kumar, D. S. Kuruvilla, Y. Shin, Y. He, J. B. Bruning, D. P. Marciano, M. D. Cameron, D. Laznik, M. J. Jurczak, S. C. Schürer, D. Vidović, G. I. Shulman, B. M. Spiegelman and P. R. Griffin (2011). Antidiabetic actions of a non-agonist PPARγ ligand blocking Cdk5-mediated phosphorylation. Nature. **477:** 477-481.

Chowdhury, M. K., N. Turner, N. L. Bentley, A. Das, L. E. Wu, D. Richani, S. Bustamante, R. B. Gilchrist, M. J. Morris, P. R. Shepherd and G. C. Smith (2017). "Niclosamide reduces glucagon sensitivity via hepatic PKA inhibition in obese mice: Implications for glucose metabolism improvements in type 2 diabetes." Sci Rep **7**: 40159.

Cooper, D. E., T. J. Grevengoed, E. L. Klett and R. A. Coleman (2015). "Glycerol-3-phosphate Acyltransferase Isoform-4 (GPAT4) Limits Oxidation of Exogenous Fatty Acids in Brown Adipocytes." J Biol Chem **290**(24): 15112-15120.

Crunk, A. E., J. Monks, A. Murakami, M. Jackman, P. S. Maclean, M. Ladinsky, E. S. Bales, S. Cain, D. J. Orlicky and J. L. McManaman (2013). "Dynamic regulation of hepatic lipid droplet properties by diet." PLoS One **8**(7): e67631.

Culp, D. J., B. Robinson, S. Parkkila, P. W. Pan, M. N. Cash, H. N. Truong, T. W. Hussey and S. L. Gullett (2011). "Oral colonization by Streptococcus mutans and caries development is reduced upon deletion of carbonic anhydrase VI expression in saliva." Biochim Biophys Acta **1812**(12): 1567-1576.

Dalle-Donne, I., R. Rossi, G. Colombo, D. Giustarini and A. Milzani (2009). "Protein S-glutathionylation: a regulatory device from bacteria to humans." Trends Biochem Sci **34**(2): 85-96.

Del Prete, S., D. Vullo, G. M. Fisher, K. T. Andrews, S. A. Poulsen, C. Capasso and C. T. Supuran (2014). "Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum--the eta-carbonic anhydrases." Bioorg Med Chem Lett **24**(18): 4389-4396.

Deol, P., J. R. Evans, J. Dhahbi, K. Chellappa, D. S. Han, S. Spindler and F. M. Sladek (2015). "Soybean Oil Is More Obesogenic and Diabetogenic than Coconut Oil and Fructose in Mouse: Potential Role for the Liver." PLoS One **10**(7): e0132672.

Ding, Y., Y. Wu, R. Zeng and K. Liao (2012). "Proteomic profiling of lipid droplet-associated proteins in primary adipocytes of normal and obese mouse." Acta Biochim Biophys Sin (Shanghai) **44**(5): 394-406.

Dodgson, S. J. (1991). "Why are there carbonic anhydrases in the liver?" Biochem Cell Biol **69**(12): 761-763.

Dodgson, S. J. and R. E. Forster, 2nd (1986). "Inhibition of CA V decreases glucose synthesis from pyruvate." Arch Biochem Biophys **251**(1): 198-204.

Dodgson, S. J., B. Quistorff and Y. Ridderstrale (1993). "Carbonic anhydrases in cytosol, nucleus, and membranes of rat liver." J Appl Physiol (1985) **75**(3): 1186-1193.

Dodgson, S. J. and M. Watford (1990). "Differential regulation of hepatic carbonic anhydrase isozymes in the streptozotocin-diabetic rat." Arch Biochem Biophys **277**(2): 410-414.

Dubuc, P. U., N. J. Wilden and H. J. Carlisle (1985). "Fed and fasting thermoregulation in ob/ob mice." Ann Nutr Metab **29**(6): 358-365.

Duda, D. M., C. Tu, S. Z. Fisher, H. An, C. Yoshioka, L. Govindasamy, P. J. Laipis, M. Agbandje-McKenna, D. N. Silverman and R. McKenna (2005). "Human carbonic anhydrase III: structural and kinetic study of catalysis and proton transfer." Biochemistry **44**(30): 10046-10053.

Dudele, A., G. M. Rasmussen, D. Mayntz, H. Malte, S. Lund and T. Wang (2015). "Effects of ambient temperature on glucose tolerance and insulin sensitivity test outcomes in normal and obese C57 male mice." Physiol Rep **3**(5).

Eakins, R., J. Walsh, L. Randle, R. E. Jenkins, I. Schuppe-Koistinen, C. Rowe, P. Starkey Lewis, O. Vasieva, N. Prats, N. Brillant, M. Auli, M. Bayliss, S. Webb, J. A. Rees, N. R. Kitteringham, C. E. Goldring and B. K. Park (2015). "Adaptation to acetaminophen exposure elicits major changes in expression and distribution of the hepatic proteome." Sci Rep **5**: 16423.

Edsall, J. T. (1984). "Some perspectives on carbonic anhydrase since 1960." Ann N Y Acad Sci **429**: 18-25.

Edwards, Y., F. Drummond and J. Sowden (2000). "Regulation of the CA1, CA2 and CA3 genes." EXS(90): 121-141.

Eisinger, K., G. Liebisch, G. Schmitz, C. Aslanidis, S. Krautbauer and C. Buechler (2014). "Lipidomic analysis of serum from high fat diet induced obese mice." Int J Mol Sci **15**(2): 2991-3002.

Elchuri, S., M. Naeemuddin, O. Sharpe, W. H. Robinson and T. T. Huang (2007). "Identification of biomarkers associated with the development of hepatocellular carcinoma in CuZn superoxide dismutase deficient mice." Proteomics **7**(12): 2121-2129.

Elchuri, S., T. D. Oberley, W. Qi, R. S. Eisenstein, L. Jackson Roberts, H. Van Remmen, C. J. Epstein and T. T. Huang (2005). "CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life." Oncogene **24**(3): 367-380.

Elder, I., Z. Fisher, P. J. Laipis, C. Tu, R. McKenna and D. N. Silverman (2007). "Structural and kinetic analysis of proton shuttle residues in the active site of human carbonic anhydrase III." Proteins **68**(1): 337-343.

Esbaugh, A. J. and B. L. Tufts (2006). "The structure and function of carbonic anhydrase isozymes in the respiratory system of vertebrates." Respir Physiol Neurobiol **154**(1-2): 185-198.

Feeney, E. L. and J. E. Hayes (2014). "Exploring associations between taste perception, oral anatomy and polymorphisms in the carbonic anhydrase (gustin) gene CA6." Physiol Behav **128**: 148-154.

Feldmann, H. M., V. Golozoubova, B. Cannon and J. Nedergaard (2009). "UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality." Cell Metab **9**(2): 203-209.

Feng, H. Z. and J. P. Jin (2016). "Carbonic Anhydrase III Is Expressed in Mouse Skeletal Muscles Independent of Fiber Type-Specific Myofilament Protein Isoforms and Plays a Role in Fatigue Resistance." Front Physiol **7**: 597.

Ferrell, J. M. and J. Y. Chiang (2015). "Short-term circadian disruption impairs bile acid and lipid homeostasis in mice." Cell Mol Gastroenterol Hepatol **1**(6): 664-677.

Forner, F., C. Kumar, C. A. Luber, T. Fromme, M. Klingenspor and M. Mann (2009). "Proteome differences between brown and white fat mitochondria reveal specialized metabolic functions." Cell Metab **10**(4): 324-335.

Frasseto, F., T. M. Parisotto, R. C. Peres, M. R. Marques, S. R. Line and M. Nobre Dos Santos (2012). "Relationship among salivary carbonic anhydrase VI activity and flow rate, biofilm pH and caries in primary dentition." Caries Res **46**(3): 194-200.

Friedman, J. M. and J. L. Halaas (1998). "Leptin and the regulation of body weight in mammals." Nature **395**(6704): 763-770.

Frost, S. C. (2014). "Physiological functions of the alpha class of carbonic anhydrases." Subcell Biochem **75**: 9-30.

Fuentealba, I. C. and E. M. Aburto (2003). "Animal models of copper-associated liver disease." Comp Hepatol **2**(1): 5.

Fuller, L. M. (1984). "The pharmacology of drinking behavior." Pharmacol Ther **24**(2): 179-206.
Geisler, C. E., C. Hepler, M. R. Higgins and B. J. Renquist (2016). "Hepatic adaptations to maintain metabolic homeostasis in response to fasting and refeeding in mice." Nutr Metab (Lond) **13**: 62.

Gibson, D. M., E. B. Titchener and S. J. Wakil (1958). "Studies on the mechanism of fatty acid synthesis. V. Bicarbonate requirement for the synthesis of long-chain fatty acids." Biochim Biophys Acta **30**(2): 376-383.

Grimes, A., J. Paynter, I. D. Walker, M. Bhave and J. F. Mercer (1997). "Decreased carbonic anhydrase III levels in the liver of the mouse mutant 'toxic milk' (tx) due to copper accumulation." Biochem J **321 (Pt 2)**: 341-346.

Gut, M. O., S. Parkkila, Z. Vernerova, E. Rohde, J. Zavada, M. Hocker, J. Pastorek, T. Karttunen, A. Gibadulinova, Z. Zavadova, K. P. Knobeloch, B. Wiedenmann, J. Svoboda, I. Horak and S. Pastorekova (2002). "Gastric hyperplasia in mice with targeted disruption of the carbonic anhydrase gene Car9." Gastroenterology **123**(6): 1889-1903.

Halmi, P., J. Lehtonen, A. Waheed, W. S. Sly and S. Parkkila (2004). "Expression of hypoxia-inducible, membrane-bound carbonic anhydrase isozyme XII in mouse tissues." Anat Rec A Discov Mol Cell Evol Biol **277**(1): 171-177.

Harju, A. K., F. Bootorabi, M. Kuuslahti, C. T. Supuran and S. Parkkila (2013). "Carbonic anhydrase III: a neglected isozyme is stepping into the limelight." J Enzyme Inhib Med Chem **28**(2): 231-239.

Harms, M. and P. Seale (2013). "Brown and beige fat: development, function and therapeutic potential." Nat Med **19**(10): 1252-1263.

Hazen, S. A., A. Waheed, W. S. Sly, K. F. LaNoue and C. J. Lynch (1996). "Differentiation-dependent expression of CA V and the role of carbonic anhydrase isozymes in pyruvate carboxylation in adipocytes." FASEB J **10**(4): 481-490.

Henkel, C., M. Roderfeld, R. Weiskirchen, M. L. Berres, S. Hillebrandt, F. Lammert, H. E. Meyer, K. Stuhler, J. Graf and E. Roeb (2006). "Changes of the hepatic proteome in murine models for toxically induced fibrogenesis and sclerosing cholangitis." Proteomics **6**(24): 6538-6548.

Herbert, J. D. and R. A. Coulson (1984). "A role for carbonic anhydrase in de novo fatty acid synthesis in liver." Ann N Y Acad Sci **429**: 525-527.

Hilvo, M., M. Rafajova, S. Pastorekova, J. Pastorek and S. Parkkila (2004). "Expression of carbonic anhydrase IX in mouse tissues." J Histochem Cytochem **52**(10): 1313-1322.

Hilvo, M., M. Tolvanen, A. Clark, B. Shen, G. N. Shah, A. Waheed, P. Halmi, M. Hanninen, J. M. Hamalainen, M. Vihinen, W. S. Sly and S. Parkkila (2005). "Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase." Biochem J **392**(Pt 1): 83-92.

Hirota, J., H. Ando, K. Hamada and K. Mikoshiba (2003). "Carbonic anhydrase-related protein is a novel binding protein for inositol 1,4,5-trisphosphate receptor type 1." Biochem J **372**(Pt 2): 435-441.

Hong, J. H., E. Muhammad, C. Zheng, E. Hershkovitz, S. Alkrinawi, N. Loewenthal, R. Parvari and S. Muallem (2015). "Essential role of carbonic anhydrase XII in secretory gland fluid and HCO3 (-) secretion revealed by disease causing human mutation." J Physiol **593**(24): 5299-5312.

Hutcheson, R. and P. Rocic (2012). "The metabolic syndrome, oxidative stress, environment, and cardiovascular disease: the great exploration." Exp Diabetes Res **2012**: 271028.

Jagoe, R. T., S. H. Lecker, M. Gomes and A. L. Goldberg (2002). "Patterns of gene expression in atrophying skeletal muscles: response to food deprivation." FASEB J **16**(13): 1697-1712.

Jewell, D. A., C. K. Tu, S. R. Paranawithana, S. M. Tanhauser, P. V. LoGrasso, P. J. Laipis and D. N. Silverman (1991). "Enhancement of the catalytic properties of human carbonic anhydrase III by site-directed mutagenesis." Biochemistry **30**(6): 1484-1490.

Jiao, Y., J. Yan, Y. Zhao, L. R. Donahue, W. G. Beamer, X. Li, B. A. Roe, M. S. Ledoux and W. Gu (2005). "Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice." Genetics **171**(3): 1239-1246.

Jitrapakdee, S., M. St Maurice, I. Rayment, W. W. Cleland, J. C. Wallace and P. V. Attwood (2008). "Structure, mechanism and regulation of pyruvate carboxylase." Biochem J **413**(3): 369-387.

Johnson, J. A., S. E. Trasino, A. W. Ferrante, Jr. and J. R. Vasselli (2007). "Prolonged decrease of adipocyte size after rosiglitazone treatment in high- and low-fat-fed rats." Obesity (Silver Spring) **15**(11): 2653-2663.

Johnson, R. F., T. G. Beltz, A. K. Johnson and R. L. Thunhorst (2015). "Effects of fludrocortisone on water and sodium intake of C57BL/6 mice." Am J Physiol Regul Integr Comp Physiol **309**(3): R247-254.

Kallio, H., M. Hilvo, A. Rodriguez, E. H. Lappalainen, A. M. Lappalainen and S. Parkkila (2010). "Global transcriptional response to carbonic anhydrase IX deficiency in the mouse stomach." BMC Genomics **11**: 397.

Kalsbeek, A. and J. H. Strubbe (1998). "Circadian control of insulin secretion is independent of the temporal distribution of feeding." Physiol Behav **63**(4): 553-558.

Kanshin, E., S. Wang, L. Ashmarina, M. Fedjaev, I. Nifant'ev, G. A. Mitchell and A. V. Pshezhetsky (2009). "The stoichiometry of protein phosphorylation in adipocyte lipid droplets: analysis by N-terminal isotope tagging and enzymatic dephosphorylation." Proteomics **9**(22): 5067-5077.

Katovich, M. J. and M. J. Fregly (1978). "Mediation of isoproterenol-induced thirst in rats by beta2-adrenergic receptors." Can J Physiol Pharmacol **56**(3): 465-470.

Khan, S. A., E. E. Wollaston-Hayden, T. W. Markowski, L. Higgins and D. G. Mashek (2015). "Quantitative analysis of the murine lipid droplet-associated proteome during diet-induced hepatic steatosis." J Lipid Res **56**(12): 2260-2272.

Kharbanda, K. K., V. Vigneswara, B. L. McVicker, A. U. Newlaczyl, K. Bailey, D. Tuma, D. E. Ray and W. G. Carter (2009). "Proteomics reveal a concerted upregulation of methionine metabolic pathway enzymes, and downregulation of carbonic anhydrase-III, in betaine supplemented ethanol-fed rats." Biochem Biophys Res Commun **381**(4): 523-527.

Kim, G., T. H. Lee, P. Wetzel, C. Geers, M. A. Robinson, T. G. Myers, J. W. Owens, N. B. Wehr, M. W. Eckhaus, G. Gros, A. Wynshaw-Boris and R. L. Levine (2004). "Carbonic

anhydrase III is not required in the mouse for normal growth, development, and life span." Mol Cell Biol **24**(22): 9942-9947.

Kim, G. and R. L. Levine (2005). "Molecular determinants of S-glutathionylation of carbonic anhydrase 3." Antioxid Redox Signal **7**(7-8): 849-854.

Kitade, H., G. Chen, Y. Ni and T. Ota (2017). "Nonalcoholic Fatty Liver Disease and Insulin Resistance: New Insights and Potential New Treatments." Nutrients **9**(4).

Kivela, J., S. Parkkila, A. K. Parkkila, J. Leinonen and H. Rajaniemi (1999). "Salivary carbonic anhydrase isoenzyme VI." J Physiol **520 Pt 2**: 315-320.

Koonen, D. P., R. L. Jacobs, M. Febbraio, M. E. Young, C. L. Soltys, H. Ong, D. E. Vance and J. R. Dyck (2007). "Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity." Diabetes **56**(12): 2863-2871.

Kopecka, J., I. Campia, A. Jacobs, A. P. Frei, D. Ghigo, B. Wollscheid and C. Riganti (2015). "Carbonic anhydrase XII is a new therapeutic target to overcome chemoresistance in cancer cells." Oncotarget **6**(9): 6776-6793.

Kopecka, J., G. M. Rankin, I. C. Salaroglio, S. A. Poulsen and C. Riganti (2016). "P-glycoprotein-mediated chemoresistance is reversed by carbonic anhydrase XII inhibitors." Oncotarget **7**(52): 85861-85875.

Lai, M., P. C. Chandrasekera and N. D. Barnard (2014). "You are what you eat, or are you? The challenges of translating high-fat-fed rodents to human obesity and diabetes." Nutr Diabetes **4**: e135.

Lau, J. K., X. Zhang and J. Yu (2017). "Animal models of non-alcoholic fatty liver disease: current perspectives and recent advances." J Pathol **241**(1): 36-44.

Le Lay, S., G. Simard, M. C. Martinez and R. Andriantsitohaina (2014). "Oxidative stress and metabolic pathologies: from an adipocentric point of view." Oxid Med Cell Longev **2014**: 908539.

Lee, M., B. Vecchio-Pagan, N. Sharma, A. Waheed, X. Li, K. S. Raraigh, S. Robbins, S. T. Han, A. L. Franca, M. J. Pellicore, T. A. Evans, K. M. Arcara, H. Nguyen, S. Luan, D. Belchis, J. Hertecant, J. Zabner, W. S. Sly and G. R. Cutting (2016). "Loss of carbonic anhydrase XII function in individuals with elevated sweat chloride concentration and pulmonary airway disease." Hum Mol Genet **25**(10): 1923-1933.

Lehenkari, P., T. A. Hentunen, T. Laitala-Leinonen, J. Tuukkanen and H. K. Vaananen (1998). "Carbonic anhydrase II plays a major role in osteoclast differentiation and bone resorption by effecting the steady state intracellular pH and Ca2+." Exp Cell Res **242**(1): 128-137.

Lehtonen, J., B. Shen, M. Vihinen, A. Casini, A. Scozzafava, C. T. Supuran, A. K. Parkkila, J. Saarnio, A. J. Kivela, A. Waheed, W. S. Sly and S. Parkkila (2004). "Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family." J Biol Chem **279**(4): 2719-2727.

Leppilampi, M., T. J. Karttunen, J. Kivela, M. O. Gut, S. Pastorekova, J. Pastorek and S. Parkkila (2005). "Gastric pit cell hyperplasia and glandular atrophy in carbonic anhydrase IX knockout mice: studies on two strains C57/BL6 and BALB/C." Transgenic Res **14**(5): 655-663.

Li, X., T. A. Gianoulis, K. Y. Yip, M. Gerstein and M. Snyder (2010). "Extensive in vivo metabolite-protein interactions revealed by large-scale systematic analyses." Cell **143**(4): 639-650.

Lii, C. K., Y. C. Chai, W. Zhao, J. A. Thomas and S. Hendrich (1994). "S-thiolation and irreversible oxidation of sulfhydryls on carbonic anhydrase III during oxidative stress: a method for studying protein modification in intact cells and tissues." Arch Biochem Biophys **308**(1): 231-239.

Lindskog, S. and D. N. Silverman (2000). "The catalytic mechanism of mammalian carbonic anhydrases." EXS(90): 175-195.

Liu, M., G. A. Walter, N. C. Pathare, R. E. Forster and K. Vandenborne (2007). "A quantitative study of bioenergetics in skeletal muscle lacking carbonic anhydrase III using 31P magnetic resonance spectroscopy." Proc Natl Acad Sci U S A **104**(1): 371-376.

LoGrasso, P. V., C. Tu, X. Chen, S. Taoka, P. J. Laipis and D. N. Silverman (1993). "Influence of amino acid replacement at position 198 on catalytic properties of zinc-bound water in human carbonic anhydrase III." Biochemistry **32**(22): 5786-5791.

LoGrasso, P. V., C. K. Tu, D. A. Jewell, G. C. Wynns, P. J. Laipis and D. N. Silverman (1991). "Catalytic enhancement of human carbonic anhydrase III by replacement of phenylalanine-198 with leucine." Biochemistry **30**(34): 8463-8470.

Lusty, C. J. (1978). "Carbamyl phosphate synthetase. Bicarbonate-dependent hydrolysis of ATP and potassium activation." J Biol Chem **253**(12): 4270-4278.

Lynch, C. J., W. A. Brennan, Jr., T. C. Vary, N. Carter and S. J. Dodgson (1993). "Carbonic anhydrase III in obese Zucker rats." Am J Physiol **264**(4 Pt 1): E621-630.

Lynch, C. J., H. Fox, S. A. Hazen, B. A. Stanley, S. Dodgson and K. F. Lanoue (1995). "Role of hepatic carbonic anhydrase in de novo lipogenesis." Biochem J **310 (Pt 1)**: 197-202.

Lynch, J., M. Mccall, L. F. Martin, L. E. E. A. Witters, S. J. Vannucci, J. Christopher, K. M. Mccall, L. Melvin, L. M. Bohlen, S. P. Hreniuk and L. A. Witters (1992). "Pyruvate carboxylase in genetic obesity."

Mabrouk, G. M., I. M. Helmy, K. G. Thampy and S. J. Wakil (1990). "Acute hormonal control of acetyl-CoA carboxylase. The roles of insulin, glucagon, and epinephrine." J Biol Chem **265**(11): 6330-6338.

Mallis, R. J., B. W. Poland, T. K. Chatterjee, R. a. Fisher, S. Darmawan, R. B. Honzatko and J. a. Thomas (2000). "Crystal structure of S-glutathiolated carbonic anhydrase III." FEBS letters **482**: 237-241.

Maury, E., K. M. Ramsey and J. Bass (2010). "Circadian rhythms and metabolic syndrome: from experimental genetics to human disease." Circ Res **106**(3): 447-462.

Meldrum, N. U. and F. J. Roughton (1933). "Carbonic anhydrase. Its preparation and properties." J Physiol **80**(2): 113-142.

Mendez, L., S. Ciordia, M. S. Fernandez, S. Juarez, A. Ramos, M. Pazos, J. M. Gallardo, J. L. Torres, M. R. Nogues and I. Medina (2017). "Changes in liver proteins of rats fed standard and high-fat and sucrose diets induced by fish omega-3 PUFAs and their combination with grape polyphenols according to quantitative proteomics." J Nutr Biochem **41**: 84-97.

Mitterberger, M. C., G. Kim, U. Rostek, R. L. Levine and W. Zwerschke (2012). "Carbonic anhydrase III regulates peroxisome proliferator-activated receptor-2." Experimental Cell Research **318**: 877-886.

Monti, D. M., G. De Simone, E. Langella, C. T. Supuran, A. Di Fiore and S. M. Monti (2017). "Insights into the role of reactive sulfhydryl groups of Carbonic Anhydrase III and VII during oxidative damage." J Enzyme Inhib Med Chem **32**(1): 5-12.

Morimoto, K., I. Nishimori, T. Takeuchi, T. Kohsaki, N. Okamoto, T. Taguchi, S. Yunoki, R. Watanabe, Y. Ohtsuki and S. Onishi (2005). "Overexpression of carbonic anhydrase-related protein XI promotes proliferation and invasion of gastrointestinal stromal tumors." Virchows Arch **447**(1): 66-73.

Nishimori, I., T. Minakuchi, S. Onishi, D. Vullo, A. Cecchi, A. Scozzafava and C. T. Supuran (2007). "Carbonic anhydrase inhibitors: cloning, characterization, and inhibition studies of the cytosolic isozyme III with sulfonamides." Bioorg Med Chem **15**(23): 7229-7236.

Nishimori, I., T. Minakuchi, S. Onishi, D. Vullo, A. Cecchi, A. Scozzafava and C. T. Supuran (2009). "Carbonic anhydrase inhibitors. Cloning, characterization and inhibition studies of the cytosolic isozyme III with anions." J Enzyme Inhib Med Chem **24**(1): 70-76.

Nishimori, I., D. Vullo, T. Minakuchi, A. Scozzafava, C. Capasso and C. T. Supuran (2013). "Restoring catalytic activity to the human carbonic anhydrase (CA) related proteins VIII, X and XI affords isoforms with high catalytic efficiency and susceptibility to anion inhibition." Bioorg Med Chem Lett **23**(1): 256-260.

Nishita, T., S. Igarashi and M. Asari (1995). "Determination of carbonic anhydrase-III by enzyme-immunoassay in liver, muscle and serum of male rats with streptozotocin-induced diabetes mellitus." Int J Biochem Cell Biol **27**(4): 359-364.

Nixon, J. P., M. Zhang, C. Wang, M. A. Kuskowski, C. M. Novak, J. A. Levine, C. J. Billington and C. M. Kotz (2010). "Evaluation of a quantitative magnetic resonance imaging system for whole body composition analysis in rodents." Obesity (Silver Spring) **18**(8): 1652-1659.

Ogilvie, J. M., K. K. Ohlemiller, G. N. Shah, B. Ulmasov, T. A. Becker, A. Waheed, A. K. Hennig, P. D. Lukasiewicz and W. S. Sly (2007). "Carbonic anhydrase XIV deficiency produces a functional defect in the retinal light response." Proc Natl Acad Sci U S A **104**(20): 8514-8519.

Okamoto, N., K. Fujikawa-Adachi, I. Nishimori, K. Taniuchi and S. Onishi (2001). "cDNA sequence of human carbonic anhydrase-related protein, CA-RP X: mRNA expressions of CA-RP X and XI in human brain." Biochim Biophys Acta **1518**(3): 311-316.

Onyenwoke, R. U., L. J. Forsberg, L. Liu, T. Williams, O. Alzate and J. E. Brenman (2012). "AMPK directly inhibits NDPK through a phosphoserine switch to maintain cellular homeostasis." Mol Biol Cell **23**(2): 381-389.

Osorio, C., P. M. Sullivan, D. N. He, B. E. Mace, J. F. Ervin, W. J. Strittmatter and O. Alzate (2007). "Mortalin is regulated by APOE in hippocampus of AD patients and by human APOE in TR mice." Neurobiol Aging **28**(12): 1853-1862.

Padiglia, A., A. Zonza, E. Atzori, C. Chillotti, C. Calo, B. J. Tepper and I. T. Barbarossa (2010). "Sensitivity to 6-n-propylthiouracil is associated with gustin (carbonic anhydrase VI) gene polymorphism, salivary zinc, and body mass index in humans." Am J Clin Nutr **92**(3): 539-545.

Patrikainen, M., P. Pan, N. Kulesskaya, V. Voikar and S. Parkkila (2014). "The role of carbonic anhydrase VI in bitter taste perception: evidence from the Car6(-)/(-) mouse model." J Biomed Sci **21**: 82.

Pinaud, R., C. Osorio, O. Alzate and E. D. Jarvis (2008). "Profiling of experience-regulated proteins in the songbird auditory forebrain using quantitative proteomics." Eur J Neurosci **27**(6): 1409-1422.

Popkin, B. M., K. E. D'Anci and I. H. Rosenberg (2010). "Water, hydration, and health." Nutr Rev **68**(8): 439-458.

Putri, M., M. R. Syamsunarno, T. Iso, A. Yamaguchi, H. Hanaoka, H. Sunaga, N. Koitabashi, H. Matsui, C. Yamazaki, S. Kameo, Y. Tsushima, T. Yokoyama, H. Koyama, N. A. Abumrad and M. Kurabayashi (2015). "CD36 is indispensable for thermogenesis under conditions of fasting and cold stress." Biochem Biophys Res Commun **457**(4): 520-525.

Quinn, C. E., P. K. Hamilton, C. J. Lockhart and G. E. McVeigh (2008). "Thiazolidinediones: effects on insulin resistance and the cardiovascular system." Br J Pharmacol **153**(4): 636-645.

Raisanen, S. R., P. Lehenkari, M. Tasanen, P. Rahkila, P. L. Harkonen and H. K. Vaananen (1999). "Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis." FASEB J **13**(3): 513-522.

Ranabir, S. and K. Reetu (2011). "Stress and hormones." Indian J Endocrinol Metab **15**(1): 18-22.

Register, A. M., M. K. Koester and E. A. Noltmann (1978). "Discovery of carbonic anhydrase in rabbit skeletal muscle and evidence for its identity with "basic muscle protein"." J Biol Chem **253**(12): 4143-4152.

Renner, S. W., L. M. Walker, L. J. Forsberg, J. Z. Sexton and J. E. Brenman (2017). "Carbonic anhydrase III (Car3) is not required for fatty acid synthesis and does not protect against high-fat diet induced obesity in mice." PLoS One **12**(4): e0176502.

Ronchi, V. P., R. D. Conde, J. C. Guillemot and P. M. Sanllorenti (2004). "The mouse liver content of carbonic anhydrase III and glutathione S-tranferases A3 and P1 depend on dietary supply of methionine and cysteine." Int J Biochem Cell Biol **36**(10): 1993-2004.

Rutkowski, J. M., J. H. Stern and P. E. Scherer (2015). "The cell biology of fat expansion." J Cell Biol **208**(5): 501-512.

Ruusuvuori, E., A. K. Huebner, I. Kirilkin, A. Y. Yukin, P. Blaesse, M. Helmy, H. J. Kang, M. El Muayed, J. Christopher Hennings, J. Voipio, N. Šestan, C. A. Hübner and K. Kaila (2013). "Neuronal carbonic anhydrase VII provides GABAergic excitatory drive to exacerbate febrile seizures." The EMBO Journal **32**(16): 2275-2286.

Ruusuvuori, E., H. Li, K. Huttu, J. M. Palva, S. Smirnov, C. Rivera, K. Kaila and J. Voipio (2004). "Carbonic anhydrase isoform VII acts as a molecular switch in the development of synchronous gamma-frequency firing of hippocampal CA1 pyramidal cells." J Neurosci **24**(11): 2699-2707.

Sahlin, K., M. Tonkonogi and K. Soderlund (1998). "Energy supply and muscle fatigue in humans." Acta Physiol Scand **162**(3): 261-266.

Sanllorenti, P. M., J. Rosenfeld, V. P. Ronchi, P. Ferrera and R. D. (2001). "Two dimensional non equilibrium pH gel electrophoresis mapping of cytosolic protein changes caused by dietary protein depletion in mouse liver." Mol Cell Biochem **220** 49-56.

Scholten, D., J. Trebicka, C. Liedtke and R. Weiskirchen (2015). "The carbon tetrachloride model in mice." Lab Anim **49**(1 Suppl): 4-11.

Shah, G. N., G. Bonapace, P. Y. Hu, P. Strisciuglio and W. S. Sly (2004). "Carbonic anhydrase II deficiency syndrome (osteopetrosis with renal tubular acidosis and brain calcification): novel mutations in CA2 identified by direct sequencing expand the opportunity for genotype-phenotype correlation." Hum Mutat **24**(3): 272.

Shah, G. N., D. Hewett-Emmett, J. H. Grubb, M. C. Migas, R. E. Fleming, A. Waheed and W. S. Sly (2000). "Mitochondrial carbonic anhydrase CA VB: differences in tissue distribution and pattern of evolution from those of CA VA suggest distinct physiological roles." Proc Natl Acad Sci U S A **97**(4): 1677-1682.

Shah, G. N., T. S. Rubbelke, J. Hendin, H. Nguyen, A. Waheed, J. D. Shoemaker and W. S. Sly (2013). "Targeted mutagenesis of mitochondrial carbonic anhydrases VA and VB implicates both enzymes in ammonia detoxification and glucose metabolism." Proc Natl Acad Sci U S A **110**(18): 7423-7428.

Shah, G. N., B. Ulmasov, A. Waheed, T. Becker, S. Makani, N. Svichar, M. Chesler and W. S. Sly (2005). "Carbonic anhydrase IV and XIV knockout mice: roles of the respective carbonic anhydrases in buffering the extracellular space in brain." Proc Natl Acad Sci U S A **102**(46): 16771-16776.

Shang, X., Y. Bao, S. Chen, H. Ren, H. Huang and Y. Li (2012). "Expression and purification of TAT-fused carbonic anhydrase III and its effect on C2C12 cell apoptosis induced by hypoxia/reoxygenation." Arch Med Sci **8**(4): 711-718.

Sheedfar, F., M. M. Sung, M. Aparicio-Vergara, N. J. Kloosterhuis, M. E. Miquilena-Colina, J. Vargas-Castrillon, M. Febbraio, R. L. Jacobs, A. de Bruin, M. Vinciguerra, C. Garcia-Monzon, M. H. Hofker, J. R. Dyck and D. P. Koonen (2014). "Increased hepatic CD36 expression with age is associated with enhanced susceptibility to nonalcoholic fatty liver disease." Aging (Albany NY) **6**(4): 281-295.

Shiels, A., S. Jeffery, C. Wilson and N. Carter (1984). "Radioimmunoassay of carbonic anhydrase III in rat tissues." Biochem J **218**(2): 281-284.

Sly, W. S. and P. Y. Hu (1995). "Human carbonic anhydrases and carbonic anhydrase deficiencies." Annu Rev Biochem **64**: 375-401.

Spicer, S. S., Z. H. Ge, R. E. Tashian, D. J. Hazen-Martin and B. A. Schulte (1990). "Comparative distribution of carbonic anhydrase isozymes III and II in rodent tissues." Am J Anat **187**(1): 55-64.

Stanton, L. W., P. A. Ponte, R. T. Coleman and M. A. Snyder (1991). "Expression of CA III in rodent models of obesity." Mol Endocrinol **5**(6): 860-866.

Sterky, F. H., J. H. Trotter, S. J. Lee, C. V. Recktenwald, X. Du, B. Zhou, P. Zhou, J. Schwenk, B. Fakler and T. C. Sudhof (2017). "Carbonic anhydrase-related protein CA10 is an evolutionarily conserved pan-neurexin ligand." Proc Natl Acad Sci U S A **114**(7): E1253-E1262.

Supuran, C. T. (2008). "Carbonic anhydrases: novel therapeutic applications for inhibitors and activators." Nat Rev Drug Discov **7**(2): 168-181.

Takahashi, H., M. Oh-Ishi, H. Shimizu and M. Mori (2001). "Detection and identification of subcutaneous adipose tissue protein related to obesity in New Zealand obese mouse." Endocr J **48**(2): 205-211.

Tao, H., Y. Zhang, X. Zeng, G. I. Shulman and S. Jin (2014). "Niclosamide ethanolamine-induced mild mitochondrial uncoupling improves diabetic symptoms in mice." Nat Med.

Thomas, J. A., B. Poland and R. Honzatko (1995). "Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation." Arch Biochem Biophys **319**(1): 1-9.

Toohey, J. I. (2014). "Sulfur amino acids in diet-induced fatty liver: a new perspective based on recent findings." Molecules **19**(6): 8334-8349.

Townsend, K. L. and Y. H. Tseng (2014). "Brown fat fuel utilization and thermogenesis." Trends Endocrinol Metab **25**(4): 168-177.

Truppo, E., C. T. Supuran, A. Sandomenico, D. Vullo, A. Innocenti, A. Di Fiore, V. Alterio, G. De Simone and S. M. Monti (2012). "Carbonic anhydrase VII is S-glutathionylated without loss of catalytic activity and affinity for sulfonamide inhibitors." Bioorg Med Chem Lett **22**(4): 1560-1564.

Tu, C., X. Chen, X. Ren, P. V. LoGrasso, D. A. Jewell, P. J. Laipis and D. N. Silverman (1994). "Interactions of active-site residues and catalytic activity of human carbonic anhydrase III." J Biol Chem **269**(37): 23002-23006.

Tu, C. K., D. N. Silverman, C. Forsman, B. H. Jonsson and S. Lindskog (1989). "Role of histidine 64 in the catalytic mechanism of human carbonic anhydrase II studied with a site-specific mutant." Biochemistry **28**(19): 7913-7918.

Tureci, O., U. Sahin, E. Vollmar, S. Siemer, E. Gottert, G. Seitz, A. K. Parkkila, G. N. Shah, J. H. Grubb, M. Pfreundschuh and W. S. Sly (1998). "Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers." Proc Natl Acad Sci U S A **95**(13): 7608-7613.

Turkmen, S., G. Guo, M. Garshasbi, K. Hoffmann, A. J. Alshalah, C. Mischung, A. Kuss, N. Humphrey, S. Mundlos and P. N. Robinson (2009). "CA8 mutations cause a novel syndrome characterized by ataxia and mild mental retardation with predisposition to quadrupedal gait." PLoS Genet **5**(5): e1000487.

van Karnebeek, C. D., W. S. Sly, C. J. Ross, R. Salvarinova, J. Yaplito-Lee, S. Santra, C. Shyr, G. A. Horvath, P. Eydoux, A. M. Lehman, V. Bernard, T. Newlove, H. Ukpeh, A. Chakrapani, M. A. Preece, S. Ball, J. Pitt, H. D. Vallance, M. Coulter-Mackie, H. Nguyen, L. H. Zhang, A. P. Bhavsar, G. Sinclair, A. Waheed, W. W. Wasserman and S. Stockler-Ipsiroglu (2014). "Mitochondrial carbonic anhydrase VA deficiency resulting from CA5A alterations presents with hyperammonemia in early childhood." Am J Hum Genet **94**(3): 453-461.

Van Schothorst, E. M., N. Franssen-van Hal, M. M. Schaap, J. Pennings, B. Hoebee and J. Keijer (2005). "Adipose gene expression patterns of weight gain suggest counteracting steroid hormone synthesis." Obes Res **13**(6): 1031-1041.

Viikila, P., A. J. Kivela, H. Mustonen, S. Koskensalo, A. Waheed, W. S. Sly, J. Pastorek, S. Pastorekova, S. Parkkila and C. Haglund (2016). "Carbonic anhydrase enzymes II, VII, IX and XII in colorectal carcinomas." World J Gastroenterol **22**(36): 8168-8177.

Waheed, A. and W. S. Sly (2014). "Membrane associated carbonic anhydrase IV (CA IV): a personal and historical perspective." Subcell Biochem **75**: 157-179.

Walden, T. B., I. R. Hansen, J. A. Timmons, B. Cannon and J. Nedergaard (2012). "Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues." Am J Physiol Endocrinol Metab **302**(1): E19-31.

Wallimann, T., M. Wyss, D. Brdiczka, K. Nicolay and H. M. Eppenberger (1992). "Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis." Biochem J **281 (Pt 1)**: 21-40.

Walther, T. C. and R. V. Farese, Jr. (2012). "Lipid droplets and cellular lipid metabolism." Annu Rev Biochem **81**: 687-714.

Wandernoth, P. M., N. Mannowetz, J. Szczyrba, L. Grannemann, A. Wolf, H. M. Becker, W. S. Sly and G. Wennemuth (2015). "Normal Fertility Requires the Expression of Carbonic Anhydrases II and IV in Sperm." J Biol Chem **290**(49): 29202-29216.

Wandernoth, P. M., M. Raubuch, N. Mannowetz, H. M. Becker, J. W. Deitmer, W. S. Sly and G. Wennemuth (2010). "Role of carbonic anhydrase IV in the bicarbonate-mediated activation of murine and human sperm." PLoS One **5**(11): e15061.

Wang, B., P. C. Chandrasekera and J. J. Pippin (2014). "Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes." Curr Diabetes Rev **10**(2): 131-145.
Wang, C. Y. and J. K. Liao (2012). "A mouse model of diet-induced obesity and insulin resistance." Methods Mol Biol **821**: 421-433.

Wang, Y., F. Quagliarini, V. Gusarova, J. Gromada, D. M. Valenzuela, J. C. Cohen and H. H. Hobbs (2013). "Mice lacking ANGPTL8 (Betatrophin) manifest disrupted triglyceride metabolism without impaired glucose homeostasis." Proceedings of the National Academy of Sciences of the United States of America **110**: 16109-16114.

Wendel, A. A., D. E. Cooper, O. R. Ilkayeva, D. M. Muoio and R. A. Coleman (2013). "Glycerol-3-phosphate acyltransferase (GPAT)-1, but not GPAT4, incorporates newly synthesized fatty acids into triacylglycerol and diminishes fatty acid oxidation." J Biol Chem **288**(38): 27299-27306.

Whittington, D. A., J. H. Grubb, A. Waheed, G. N. Shah, W. S. Sly and D. W. Christianson (2004). "Expression, assay, and structure of the extracellular domain of murine carbonic anhydrase XIV: implications for selective inhibition of membrane-associated isozymes." J Biol Chem **279**(8): 7223-7228.

Winzell, M. S. and B. Ahren (2004). "The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes." Diabetes **53 Suppl 3**: S215-219.

Wong, L. L., S. T. Fan, K. Man, W. H. Sit, P. P. Jiang, I. W. Jor, C. Y. Lee, W. L. Ling, K. T. Tam and J. M. Wan (2011). "Identification of liver proteins and their roles associated with carbon tetrachloride-induced hepatotoxicity." Hum Exp Toxicol **30**(9): 1369-1381.

Yamada, M., M. Satoh, M. Seimiya, K. Sogawa, S. Itoga, T. Tomonaga and F. Nomura (2013). "Combined proteomic analysis of liver tissue and serum in chronically alcohol-fed rats." Alcohol Clin Exp Res **37 Suppl 1**: E79-87.

Yamamoto, T., R. Kikkawa, H. Yamada and I. Horii (2006). "Investigation of proteomic biomarkers in in vivo hepatotoxicity study of rat liver: toxicity differentiation in hepatotoxicants." J Toxicol Sci **31**(1): 49-60.

Yang, G. Z., L. Hu, J. Cai, H. Y. Chen, Y. Zhang, D. Feng, C. Y. Qi, Y. X. Zhai, H. Gong, H. Fu, Q. P. Cai and C. F. Gao (2015). "Prognostic value of carbonic anhydrase VII expression in colorectal carcinoma." BMC Cancer **15**: 209.

Zagani, R., W. El-Assaad, I. Gamache and J. G. Teodoro (2015). "Inhibition of adipose triglyceride lipase (ATGL) by the putative tumor suppressor G0S2 or a small molecule inhibitor attenuates the growth of cancer cells." Oncotarget **6**(29): 28282-28295.

Zhang, F., X. Xu, B. Zhou, Z. He and Q. Zhai (2011). "Gene expression profile change and associated physiological and pathological effects in mouse liver induced by fasting and refeeding." PloS one **6**: e27553.

Zhou, P. (2004). "Determining protein half-lives." Methods Mol Biol **284**: 67-77.

Zimmerman, U. J., P. Wang, X. Zhang, S. Bogdanovich and R. Forster (2004). "Anti-oxidative response of carbonic anhydrase III in skeletal muscle." IUBMB Life **56**(6): 343-347.