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Concurrent overexpression of SIRT1 and knockout of GCN5 in adult skeletal muscle does not alter mitochondrial function or insulin sensitivity in mice

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by

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Abstract of the Thesis

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The NAD^+ dependent protein deacetylase sirtuin 1 (SIRT1) and the acetyltransferase general control of amino acid synthesis 5 (GCN5) are proposed to regulate glucose homeostasis and mitochondrial biogenesis through their reciprocal regulation of the acetylation status of peroxisome proliferator activated receptor-γ coactivator-α (PGC1α). However, the precise contribution of these two enzymes to glucose homeostasis and mitochondrial biogenesis in skeletal muscle, remains to be fully defined. To address this gap in knowledge, this Thesis investigated whether overexpression of SIRT1 and knockout of GCN5 in skeletal muscle would enhance glucose homeostasis and mitochondrial biogenesis. To do this, we used Cre-LoxP methodology to generate double transgenic (dTG) mice with inducible, skeletal muscle-specific overexpression of SIRT1 and knockout of GCN5. We assessed skeletal muscle insulin sensitivity by 2-deoxy-glucose uptake, glucose tolerance via an oral glucose tolerance

test, acute exercise capacity, and muscle maximal respiratory function by high-resolution respirometry and compared findings in dTG mice to those in wildtype littermate controls (WT). As expected, there was robust overexpression of SIRT1 and knockout of GCN5 in skeletal muscle of dTG compared to WT mice, and this was accompanied by increased gene expression of some mitochondrial proteins and citrate synthase activity in dTG versus WT mice. Despite these changes, there was no genotype difference in maximal respiratory function of skeletal muscle. Moreover, oral glucose tolerance and skeletal muscle insulin sensitivity were comparable between genotypes. Taken together, these results demonstrate that concurrent overexpression of SIRT1 and knockout of GCN5 does not impact skeletal muscle glucose homeostasis or mitochondrial function.

Introduction

The acetylation status of histones and other proteins controls metabolic processes such as glycolysis, gluconeogenesis, fatty acid metabolism and mitochondrial biogenesis in various tissues(Zhao et al. 2010),(Lundby et al. 2012),(Q. Wang et al. 2010). Acetylation status of proteins is dependent on abundance of nicotinamide-adenine dinucleotide (NAD^+) and acetyl-CoA(Philp et al. 2014),(Wagner and Payne 2013), with NAD^+ being the primary substrate for the sirtuin class of deacetylases (KDAC) which act to remove acetyl groups from lysine residues(Haigis and Sinclair 2010), while acetyltransferases (KAT) use acetyl-CoA as their substrate to add acetyl groups to their targets(Friedmann and Marmorstein 2013).

Peroxisome proliferator activated receptor-γ coactivator-α (PGC1α) plays an important role in mitochondrial biogenesis, controls the expression of genes involved in various metabolic pathways, and enhances glycemic control(Cantó et al. 2009; Rodgers et al. 2005; Yoon et al. 2001; Dominy et al. 2010; Coste et al. 2008; D. Lee and Goldberg 2015; Lerin et al. 2006). The transcriptional activity of PGC1α is regulated through reversible lysine acetylation by SIRT1 and GCN5, which remove and add acetyl groups, respectively(Rodgers et al. 2005; Gerhart-Hines et al. 2007; Lerin et al. 2006). Specifically, SIRT1 activates PGC1α through deacetylation(Rodgers et al. 2005; Gerhart-Hines et al. 2007; Cantó et al. 2009), while GCN5 represses PGC1α activity by acetylation(Philp et al. 2011; Bhatt, Thomas, and Nanjan 2012; Lerin et al. 2006; Coste et al. 2008). Whole body overexpression of SIRT1 in mice enhances glucose metabolism(Banks et al. 2008), whilst pharmacological activation of SIRT1 via SRT1720 improves glycemic control and insulin sensitivity in mice(Feige et al. 2008; Milne et al. 2007). In line with these observations, pharmacological activation of SIRT1 in mice during 9 weeks of exercise training increased

mitochondrial adaptations in skeletal muscle.(Menzies et al. 2013) Pharmacological activation of SIRT1 in mice was also associated with changes towards an oxidative fiber type as well as an increase in expression of PGC1α targets involved in fatty acid oxidation(Feige et al. 2008). On the other hand, increased GCN5 activity has been linked to repression of mitochondrial and fatty acid oxidation gene expression, decreased insulin stimulated glucose uptake, and decreased hepatic glucose secretion(Gerhart-Hines et al. 2007; Lerin et al. 2006; Kelly et al. 2009; Lingdi Wang et al. 2017). Related to this, following a bout of acute endurance exercise, deacetylation of PGC1α occurs in conjunction with a reduction of the nuclear prescence of GCN5 as well as its association with PGC1α; this relationship was maintained with or without SIRT1 activity(Philp et al. 2011).

To better understand the role of SIRT1 and GCN5 in skeletal muscle metabolic adaptations, we previously generated mouse models with either muscle-specific SIRT1 overexpression (mOX)(A. T. White et al. 2013; Amanda T. White et al. 2014), inducible muscle-specific SIRT1 overexpression (i-mOX)(K. Svensson et al. 2017), or a muscle-specific GCN5 knockout (mKO)(Dent et al. 2017). Interestingly, we found changes in the abundance of proteins that are a part of the mitochondrial electron transport chain in our mOX model, but not the i-mOX model or the mKO model(Amanda T. White et al. 2014; K. Svensson et al. 2017; Dent et al. 2017). Glucose homeostasis was not changed in either of the SIRT1 mouse models(K. Svensson et al. 2017; Amanda T. White et al. 2014). There were also no differences in voluntary wheel running in the mKO mice(Dent et al. 2017). A reason for these contrasting findings in vivo could be that with increased SIRT1 expression GCN5 may act to sequester more PGC1α in the nucleus, stopping any changes in transcriptional activity; and with decreased GCN5 expression SIRT1 activity may also decrease, not to overcompensate. Because no studies have been investigated the concurrent interplay of these two enzymes in adult skeletal muscle, we generated a inducible, skeletal muscle-

specific mouse model with both overexpression of SIRT1 and knockout of GCN5 (dTG). We hypothesized that these mice would exhibit enhanced skeletal muscle mitochondrial function and insulin sensitivity.

Review of Literature

Acetylation:

Post-translational modifications (PTM) have been a large area of study since their discovery as a mechanism regulating biological functions. PTM such as phosphorylation, acetylation, methylation, and ubiquitination have been studied on a global scale and have been shown to regulate cellular processes such as growth, cell cycle regulation, autophagy, and apoptosis in response to environmental stresses; with most of the focus in the past coming on phosphorylation because of the ease of detection(Philp et al. 2014). There have been over 6000 phosphorylation sites found located on 2200 proteins, as well as 500 known protein kinases, which transfer the γ-phosphate from ATP to a target protein (generating ADP), and 140 protein phosphatases, which remove the phosphate group (Hunter 2007). Acetylation has been an area of intense study since Allfrey et al. (ALLFREY, FAULKNER, and MIRSKY 1964) observed that the acetylation status of core histone tails correlated with RNA synthesis rates, which pioneered the field of acetylation in the regulation of gene transcription. It was later found that actively transcribed DNA sequences had an enrichment of acetylated histones compared to DNA sequences not actively transcribed, showing a functional role for this modification(Roth, Denu, and Allis 2001). Histone tails protrude from the chromatin polymer, serving as a platform for protein interactions which remodel the chromatin structure (Roth, Denu, and Allis 2001),(Hecht et al. 1995). The change in charge of histone tails by acetylation weakens the contact between histones and DNA(ALLFREY, FAULKNER, and MIRSKY 1964) as well as histones and regulatory proteins(Hecht et al. 1995). These modifications lead to a change in the folding of

nucleosomes, providing a more transcriptionally permissive environment in chromatin(Roth, Denu, and Allis 2001).

Acetylation has been shown to be an important PTM in the regulation of metabolism, Wang et al.(Q. Wang et al. 2010) studied in the prokaryote *Salmonella enterica* which only contains one acetyltransferase, Pat, and one deacetylase, CobB, this allowed them to observe the balance between the two under varying conditions. Wang et al. exposed *S. enterica* to glucose, inducing a glycolysis-dependent metabolic state, or citrate, inducing an oxidative/gluconeogenic metabolic state, and performed SILAC, which is a mass spectrometry method used to determine the difference in protein abundance, to determine global acetylation changes. With this method 15 enzymes with increased acetylation were identified in the glucose condition, linking carbon availability to differences in acetylation.(Q. Wang et al. 2010) Further testing on metabolic flux in different conditions on *S. enterica* showed that glucose-mediated acetylation was linked to increased glycolysis/gluconeogenesis while citrate mediated deacetylation was correlated with an increase in glyoxylate/TCA flux.(Q. Wang et al. 2010) These were the first tests showing evidence of the sensitivity of reversible acetylation to substrate availability, and they connected acetyltransferase and deacetylase activity to the utilization of distinct metabolic pathways.

The discovery of the role of acetylation in the metabolic regulation of lower organisms lead Zhao et al.(Zhao et al. 2010) to study protein acetylation in human liver samples, where they found 1,300 acetylated peptides which matched with 1,047 human proteins. Remarkably almost every enzyme involved in glycolysis, gluconeogenesis, the TCA cycle, the urea cycle, fatty acid metabolism, and glycogen metabolism were acetylated(Zhao et al. 2010). In an analysis of a rat tissue library (liver, spleen, pancreas, muscle, skin, thymus, kidney, brown fat, brain, intestine, heart, lung, stomach, testis fat, testis) it was observed that each of these tissues had about 1,000

acetylated proteins, and that tissues with a higher metabolic capacity had a higher number of acetylated proteins(Lundby et al. 2012). Lundby et al.(Lundby et al. 2012) also showed that patterns of acetylation in skeletal muscle were reflective of major energy consuming processes, there was a significant enrichment of acetylation on proteins involved in muscle contraction and metabolic function. It was also found that acetylated proteins mainly reside in the cytoplasm or nucleus (30% each) while mitochondria and the plasma membrane contain about 15% of the acetylated proteins each(Lundby et al. 2012); this differs vastly from phosphorylated proteins, which are twice as prevalent as acetylated proteins in the plasma membrane, but only a third as prevalent in mitochondria(Philp et al. 2014).

The acetylation status of a protein reflects the balance of acetyltransferase and deacetylase activity at target lysine residues, making it important to understand what controls this balance. Acetylation status of both mitochondrial and nuclear proteins is dependent on nonenzymatic factors which rely on acetyl-CoA abundance as well as fluctuations in metabolic flux(Wagner and Payne 2013). Acetyltransferases use acetyl-CoA as a substrate, whereas class III deacetylases (sirtuins) are NAD^+ dependent; demonstrating that KAT/KDAC activity is dependent on substrate flux through glucose, lipid, and ketogenic pathways(Philp et al. 2014) and that activity is reflective of cellular energy status(Wagner and Payne 2013). One example of the close connection between cellular energy status and acetylation can be found during high-energy states, where mitochondrial acetyl-CoA production is elevated. The high levels of acetyl-CoA in the mitochondria leads to non-enzymatic acetylation of mitochondrial proteins, thereby acting as a feedback mechanism, reducing enzymatic activity and decreasing substrate oxidation. Additionally, excess nuclear acetyl-CoA during high-energy states increases expression of genes

controlling energy storage and cell cycle progression, thereby connecting cellular energy state to proliferation (Philp et al. 2014; Wagner and Payne 2013).

There are five classes of histone deacetylases, which are defined as proteins with intrinsic enzymatic activity to remove acetyl groups from lysine residues. The five classes (I,IIa,IIa,III,IV) are further divided into two subclasses; classical, which is composed of classes I,IIa,IIb, and IV, and the Sir2 family of NAD^+ dependent enzymes, composed of class III deacetyltransferases(Philp et al. 2014). Sirtuins are class III deacetylases which require NAD^+ to catalyze the removal of an acetyl group from lysine residues and can also act as ADP-ribosyltransferases. There have been seven mammalian SIRTs (SIRT 1-7) identified, all of which contain a conserved NAD^+ binding site and catalytic domain, termed the core domain(Haigis and Sinclair 2010). There are four classes of SIRTs; class I consists of SIRT1-3, class II consists of SIRT4, class II of SIRT5, and class IV of SIRT6-7(Philp et al. 2014). SIRT1 and 6 are predominantly located in the nucleus, SIRT7 is nucleolar, SIRT3-5 are mitochondrial, and SIRT2 is cytoplasmic; but this does not mean they will always only be found in these locations(Philp et al. 2014).

Along with the five classes of histone deacetylases, there are two families of acetyltransferases; the GCN5 N-acetyltransferase (GNAT) family, which contains GCN5, PCAF, as well as many other, as well as the MYST HAT family. Acetyltransferases form multiple-subunit complexes, and the composition of these complexes is essential to the substrate specificity as well as the biological activity of the acetyltransferases inside(K. K. Lee and Workman 2007). Acetyltransferases belonging to the GNAT family have the common feature of transferring acetyl groups from acetyl-CoA to a primary amino group, but the different enzymes transfer to different groups(Friedmann and Marmorstein 2013). Structural analysis of the GNAT

family has shown the presence of a conserved core, which includes a binding site for acetyl-CoA used in the transfer of an acetyl group(Friedmann and Marmorstein 2013). These enzymes have been shown to be located in the nucleus in both humans as well as yeast(Friedmann and Marmorstein 2013); this makes sense as acetyltransferases have been shown to control transcriptional activity.

In all, the evidence for a role of acetylation in shaping transcription as well as mediating metabolic flux is astounding. Acetyltransferases and deacetylases help to keep the body in a homeostatic state, thus observing the interplay of two of these enzymes on a common target may help to elucidate a path to metabolic reshaping; as observing the activity of one enzyme over the other may not paint the whole picture. Two such enzymes are SIRT1 and GCN5, which we will be going in depth on.

SIRT1:

The Sir2 gene was first discovered in *Saccharomyces cerevisiae* for its role in the transcriptional silencing of mating-type loci in the budding yeast(Smith and Boeke 1997); the protein translated from this gene is the founding member of the sirtuin class of NAD^+ dependent deacetylases(Frye 1999). A product of SIRT1's NAD^+-dependent deacetylation of its targets is nicotinamide (NAM) which is a noncompetitive inhibitor of SIRT1 and inhibits its activity(Bitterman et al. 2002). This creates a negative feedback loop in which SIRT1 is inhibited by its own product, this is useful for the body to be able to revert back to anabolic pathways when nutrient stores are replenished. SIRT1 is known to regulate over 40 proteins through its deacetylase activity(Nogueiras et al. 2012). Since the discovery that an extra copy of the Sir2 gene extended life span in yeast(Kaeberlein, McVey, and Guarente 1999) there has been

increased examination on whether or not this effect could be seen in mammals. Research on the role of SIRT1 in calorie restriction, obesity and T2D, and exercise adaptations in skeletal muscle, as well as upstream and downstream targets of SIRT1 have driven forward our understanding, but it is clear that we still do not fully understand how SIRT1 contributes to these pathways.

SIRT1's ability to respond to fluctuations in cellular substrate availability by shifting muscle metabolism, through its sensitivity to changes in NAD^+, has led to a spike in studying AMP-activated protein kinase's (AMPK) role in modulating SIRT1 activity as well as the effect of SIRT1's regulation of transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α). AMPK plays a role in skeletal muscle as an energy sensor, its activity is elevated by an increase in AMP(Suter et al. 2006) which is indicative of cellular energy stress that can come from exercise(Cantó et al. 2009) or fasting[18]. When activated, AMPK is phosphorylated on Thr172(Cantó et al. 2010), and inhibits anabolic pathways that consume ATP and activates catabolic pathways in order to generate ATP(Suter et al. 2006) . Another one of its targets is Nampt, the rate limiting enzyme in the NAD^+ biosynthetic salvage pathway(Fulco et al. 2008), which when activated leads to an increase in NAD^+[18]. AIRCAR, an AMPK activator, has also been shown to increase intracellular NAD^+ levels in skeletal muscle as well as C2C12 myotubes, which in turn indirectly increases SIRT1 activity, leading to PGC1α deacetylation[17]. In agreement with this, fasting did not induce a change in NAD^+ levels in AMPK knockout mice(Cantó et al. 2010). The changes induced by AMPK are essential for metabolic adaptations in times of energy stress and plays a major role in the regulation of SIRT1's ability to deacetylate its downstream target PGC1α through its modulation of NAD^+ levels.

SIRT1 was first linked to mitochondrial adaptation through the identification of its interaction with transcriptional coactivator PGC1α, in which a complex is formed between the

two and PGC1α is deacetylated(Nemoto, Fergusson, and Finkel 2005). Rogers et al. discovered that there were thirteen lysine residues that spanned the length of the protein and underwent reversible acetylation(Rodgers et al. 2005). When PGC1α is deacetylated and active, it has been shown to affect many different aspects of metabolism, such as hepatic glucose output as well as different substrate utilization genes(Yoon et al. 2001). Through studies in cells as well as mice it was discovered that PGC1α controls the expression of genes involved in gluconeogenesis, glycolysis, lipogenesis, peroxisomal and mitochondrial fatty acid oxidation, mitochondrial respiratory efficiency, mitochondrial biogenesis(Dominy et al. 2010),[23–25],(Coste et al. 2008; D. Lee and Goldberg 2015),(Cantó et al. 2009). A whole-body overexpression of SIRT1 in mice lead to an increase in the expression of PGC1α along with other mitochondrial genes, this was also coupled with an increase in the state 3 respiration of mitochondria(Dominy et al. 2010). Contrary to that study, an overexpression of SIRT1 in rat skeletal muscle lead to a decrease in mitochondrial respiration, enzyme activity, and PGC1α protein content(Gurd et al. 2009). Temporal overexpression of SIRT1 in mouse skeletal muscle had no effect on insulin sensitivity, glucose tolerance, or markers of mitochondrial biogenesis; it was also found that the increase in SIRT1 protein did not correlate to an increase in PGC1α abundance in skeletal muscle(K. Svensson et al. 2017). In accordance with AMPK and SIRT1 activation, PGC1α is up-regulated during times of cellular energy stress such as fasting and exercise. In mice that underwent acute fasting, protein levels of PGC1α as well as mRNA of downstream gluconeogenic targets were increased in the liver, PGC1α was deacetylated in the liver as well as skeletal muscle, and the activation of the genes correlated with the length of the fast[25],(Gerhart-Hines et al. 2007). These studies demonstrated that SIRT1 deacetylation of PGC1α is activated in low nutrient conditions when other fuels must be used by the cell.

PGC1α activity also plays a role in adaptations due to exercise as well, although the role of SIRT1 is not as clear as most experiments were done *in vitro* as opposed to intact skeletal muscle. Exercise training leads to an increase in mitochondrial enzymes (ALA synthase, citrate synthase, COX subunit 1, cytoC) that was coupled with a comparable increase in PGC1α(Baar et al. n.d.),(Akimoto et al. 2005). In line with this data, a muscle specific PGC1α knockout mouse had smaller increases in mitochondrial enzymes as well as reduced angiogenesis after endurance training(Geng et al. 2010). To check if SIRT1 had a role in any of these exercise adaptations, mice with a muscle specific knockout of SIRT1 were used in an exercise study(Philp et al. 2011). Surprisingly, there was no detriment to basal mitochondrial function and gene expression due to the loss of SIRT1 activity(Philp et al. 2011). A study in rats found that after 12 weeks of training SIRT1 protein was increased, while in a human study 6 weeks of high intensity interval training increased SIRT1 activity but this was coupled with a decrease in SIRT1 protein these results may be able to explain why SIRT1 doesn't have an effect on exercise adaptations(Gurd et al. 2010; Huang et al. 2016). Next in order to test if these mice were able to adapt to exercise, both acute and chronic exercise were performed by the mice and alterations in mitochondrial content, function, PGC1α signaling, and pathways that were thought to be essential to mitochondrial adaptation were checked for. Not only did the knockout mice adapt similarly to the wildtype mice, deacetylation of PGC1α was unchanged between the groups(Philp et al. 2011).. While this may mean that SIRT1 does not play a role in the deacetylation of PGC1α due to exercise, it may also mean that PGC1α is being less acetylated during exercise highlighting the importance of researching the balance between deacetylases and acetyltransferases on their targets.

Calorie restriction (CR) has long been an area of research and has been shown to provide many health benefits including increased life span in flies, worms, and mice as well as improved

glucose homeostasis as evidenced by decreased insulin and glucose levels(Haigis and Sinclair 2010). These health benefits have generated large interest into whether SIRT1 plays a role in mammalian calorie restriction. In rats, CR was found to lead to an upregulation of SIRT1 protein expression in the brain, kidney, liver, white adipose tissue, and skeletal muscle(Cohen et al. 2004). In another study done on mice, calorie restriction increased SIRT1 as well as NAD^+ in white adipose tissue and skeletal muscle, but did not induce any changes in the liver, this led Chen et al. to create a SIRT1 liver knockout mouse model; which showed no differences in blood glucose, blood insulin, glucose tolerance, weight loss, or fat reduction when compared to the wildtype on a CR diet(Chen et al. 2008). A whole body knockout of SIRT1 in mice led to no increase in activity due to CR, while overexpression in adipose tissue and the brain (but not liver or muscle) produced mice that were leaner, were more glucose tolerant, had lower blood cholesterol, and lower insulin levels; all of which are phenotypes of CR(Chen et al. 2005; Bordone et al. 2007). In skeletal muscle, a muscle specific SIRT1 knockout in mice demonstrates that SIRT1 plays a role in enhancing insulin sensitivity brought on by CR as evidenced by better glucose disposal rate as well as higher levels of insulin stimulated 2DOGU in wildtype mice when compared to the knockouts(Schenk et al. 2011). These studies come together to show that SIRT1 does play a role in the metabolic adaptations that are associated with a calorie restricted diet, but the exact tissue that SIRT1 acts in has yet to be determined. More tissue specific knockouts should be tested to try to discover where this takes place.

The rates of obesity and diabetes in the world have been increasing over time, and because SIRT1 has demonstrated that it is able to alter insulin sensitivity as well as glucose tolerance via its activity, it makes sense to test the effects of SIRT1 on a high fat diet ,(HFD) which mimics a westernized diet, as well as on both a Type 1 and Type 2 mouse model of

diabetes. In a gain of function model, SIRT1 protects from HFD induced metabolic problems as well as problems arising in a diabetes mouse model; blood glucose and plasma insulin were lowered and there was no increase in islet B cell mass, insulin content, and section from pancreatic islets; meaning that the mice were more insulin sensitive(Banks et al. 2008). The same mice were shown to have higher levels of adiponectin, an AMPK activator, when compared to control mice(Banks et al. 2008). However mice with an overexpression of SIRT1 in skeletal muscle did not have any changes in insulin sensitivity, glucose tolerance, or insulin signaling in response to a supraphyiological insulin dose(A. T. White et al. 2013). Mice with a liver specific knockout of SIRT1 had reduced accumulation of body fat on a HFD and were more glucose tolerant and had lower levels of blood glucose and insulin, showing improved insulin sensitivity[34]. Mice with a moderate whole-body overexpression were shown to be slightly protected from HFD induced glucose intolerance(P. T. Pfluger et al. 2008). In contrast to this, studies that have looked at overexpression of SIRT1 in bona fide skeletal muscle and C2C12 myotubes have shown that a HFD still induces regular weight gain as well as impairments in insulin stimulated glucose uptake and the insulin signaling pathway(Amanda T. White et al. 2014)·(Sun et al. 2007). In the same way that there seems to be tissue specificity for SIRT1 to induce changes caused by calorie restriction, it seems as the protective effects of SIRT1 from HFD induce glucose intolerance are tissue specific as well.

Resveratrol is a naturally occurring antioxidant that activates SIRT1 *in vivo*, leading to the deacetylation of PGC1α (Baur et al. 2006; Lagouge et al. 2006). This compound may be of great interest because of its ability to activate SIRT1, and the implications it may have in the treatment of metabolic disorders such as T2D(Kristoffer Svensson, Handschin, and Christoph n.d.). Resveratrol was used on mice fed a HFD to determine whether this pathway could be used

to treat diseases, and it was observed that mice on a HFD had improved exercise performance, alleviated metabolic dysfunction, as well as extended lifespan(Baur et al. 2006; Lagouge et al. 2006). In another study resveratrol was shown to induce further mitochondrial adaptations in mice skeletal muscle in response to exercise and this response was dependent on skeletal muscle SIRT1(Dolinsky et al. 2012; Menzies et al. 2013). The results of studies like these showing the benefits of resveratrol supplementation on metabolic health in mice pushed researched to clinically test this compound on obese individuals and individuals with T2D. It was found in obese individuals, oral doses of resveratrol did not affect body weight or insulin sensitivity(Poulsen et al. 2013), but it was also shown that obese humans supplemented with resveratrol for 30 days had induced mild metabolic adaptations as well as mitochondrial gene transcription in muscle(Timmers et al. 2011). However, contrary to the results in obese patients, type 2 diabetic patients that were administered resveratrol were shown to have improved glycemic control and insulin sensitivity(Brasnyó et al. 2011; Bhatt, Thomas, and Nanjan 2012). It seems as though resveratrol treatment may be a possible way to combat type 2 diabetes, but more testing must be done in order to make sure.

GCN5:

The ability of SIRT1 to induce so many changes by deacetylating its target, in this case PGC1α makes it interesting to study the effects of GCN5 as it is the specific acetyltransferase for PGC1α and counteracts the activity of SIRT1 to acetylate and inhibit PGC1α. GCN5 was initially identified in yeast as a transcriptional regulator with high sequential identity with the catalytic subunit of p55(Brownell et al. 1996). As with most acetyltransferases GCN5 creates a complex with other proteins to exhibit its acetyltransferase activity(Lerin et al. 2006). In the case

of GCN5, the major complex that forms is the SAGA complex; which contains a histone acetylation center that contains GCN5 together with Ada proteins and a deubiquitination molecule(Li Wang and YR Dent 2014). SAGA's major substrates have been identified as several acetylation sites on H3(Li Wang and YR Dent 2014). GCN5's activity is positively regulated by steroid receptor coactivator protein (SRC-3) via specific binding directly to the promotor region, any changes in the expression of SRC-3 are correlated with the same change of expression in GCN5(Dominy et al. 2010),(Coste et al. 2008). There have been 34 target proteins identified for GCN5, with a strong overlap in targets with the sirtuin class of deacetylases(Downey et al. 2015). GCN5 has been identified to play a major role in development and maintenance, as well as nutrient homeostasis through acetylation of its targets.

GCN5 has been shown to be incredibly important in the developmental cycle, as deletion of GCN5 results in early embryonic lethality in mice due to the inability of the mesoderm to form(Roth, Denu, and Allis 2001),(Xu et al. 2000). It has been shown many times that normal GCN5 expression and activity levels are needed for proper development. Mice with a catalytically inactive GCN5 allele die in mid gestation and have severe neural tube closure defects(Bu et al. 2007). When testing the ability of GCN5 null embryonic stem cells (ES) to grow and differentiate, it was discovered that these cells could differentiate normally, however transcription factors that are pivotal in ES identity (Oct 4 and Nodal) were lost prematurely suggesting a role for GCN5 in the maintenance of pluripotent ES cells(Lin et al. 2007). However along with maintenance, a role for GCN5 in muscular atrophy has also been uncovered, as inhibition of GCN5 lowers the acetylation of the p65 subunit of NF-kB; this led to diminished muscular atrophy upon food deprivation(D. Lee and Goldberg 2015). In the same study it was found that GCN5 inhibited Akt as well as mTOR(D. Lee and Goldberg 2015). In all, past

research has shown a major role for GCN5 in the developmental stage as well as for maintenance of muscle.

GCN5 plays a major role in nutrient homeostasis through its delicate interplay with SIRT1 on the acetylation status of PGC1α, which may be important to look at as a whole system as opposed to past studies that focused on one of these enzymes. GCN5 acts as the specific acetyltransferase for PGC1α, although PCAF has been shown to acetylate it as well but to a much lesser extent(Lerin et al. 2006). In PCAF knockout mice, it was found that GCN5 protein levels were drastically elevated in tissues where PCAF is normally expressed, this may mean that PCAF is redundant in function when it comes to acetylation of PGC1α(Yamauchi et al. 2000). GCN5 physically interacts with PGC1α to acetylate and repress its activity in high nutrient conditions, after feeding, and effectively sequesters it away from its targets not allowing an increase in transcriptional activity[57]. In exercised mice GCN5 was shown to disassociate with PGC1α and leave the nucleus; this may add to how deacetylation of PGC1α occurs as opposed to direct deacetylation by SIRT1 exclusively(Philp et al. 2011). The effect this has is repression of PGC1α's positive effects on mitochondrial and fatty acid oxidation enzyme gene expression in C2C12 myotubes, repression of the effect of PGC1β on insulin stimulated glucose uptake in HEK cells, and inhibition of hepatic glucose secretion(Gerhart-Hines et al. 2007; Kelly et al. 2009; Lerin et al. 2006). In a study where GCN5 was knocked out of the muscle, there were no phenotype differences when compared to a wildtype; no changes in cellular respiration, mitochondrial protein abundance, exercise induced mitochondrial biogenesis, insulin sensitivity, glucose tolerance, body mass, fat mass, or muscle fiber composition(Dent et al. 2017). The interplay between GCN5 and SIRT1 may be able to explain why there is no effect seen in these mice.

Conclusion:

Acetylation is a powerful PTM acting on a global scale to modulate the activity of DNA transcription, transcription factors, apoptosis, nutrient homeostasis, as well as many more. SIRT1 has been identified to play a major role in substrate control, mitochondrial biogenesis, and in some cases modulation of glucose uptake. It is observed that SIRT1 deacetylates and activates PGC1α under nutrient-deprived conditions to increase fatty acid oxidation, mitochondrial biogenesis, etc. GCN5 acts to counterbalance the effect of SIRT1 by acetylating PGC1α and sequestering it away from its targets in the nucleus. Although it has been shown that SIRT1 deacetylates PGC1α directly, the disassociation and translocation of GCN5 out of the nucleus during exercise may explain why SIRT1 is not needed for exercise adaptations and may reveal a delicate balance between enzymatic activity and physical interaction to compensate for lost KAT/KDAC activity. This may be the reason for the discrepancies between phenotypes in different tissue specific models. For this reason, it is important to see how a knockout/over-expresser model of these enzymes in skeletal muscle can affect mitochondrial biogenesis, exercise capacity, and other downstream and upstream markers such as NAD^+ levels, AMPK activation, and PGC1α acetylation. This study helps us understand how these two enzymes affect acetylation in skeletal muscle, and the effects on mitochondrial biogenesis, glucose homeostasis, and exercise capacity that come with manipulation of both enzymes. We found that although there is increased transcription of mitochondrial genes and fatty acid oxidation genes, these changes do not lead to any functional mitochondrial or metabolic adaptations. Our study has helped identify that skeletal muscle is not where these enzymes exhibit their beneficial metabolic changes.

Material and Methods

Animals

We generated mice with inducible, skeletal muscle-specific overexpression of SIRT1 and knockout of GCN5 (dTG) mice by breeding mice harboring loxP sites flanking both a stop element upstream of the *Sirt1* gene(Firestein et al. 2008) and exons 3-19 of GCN5(Lin et al. 2007) with mice carrying Cre-recombinase (Cre) under the control of the human α-skeletal actin (HSA) promoter in a tamoxifen (TMX)-inducible manner(McCarthy et al. 2012). For all experiments,

male dTG and Cre-negative littermates (WT) were administered TMX via oral gavage between 5-6 weeks of age for 5 consecutive days. Mice were housed on a 12:12 h light-dark cycle, with all experiments being conducted 4–7 weeks after initiating TMX treatment. All experiments were approved and conducted in accordance with the Animal Care Program at the University of California, San Diego.

Tissue collection and body composition

Tissues were excised from fasted (4h) and anesthetized mice. Skeletal muscles (gastrocnemius [GA], quadriceps [QUAD], tibialis anterior [TA], plantaris [PLN]), heart, liver, and epididymal adipose tissue (eWAT) were rinsed in sterile saline, blotted dry, weighed, and frozen in liquid nitrogen. All tissues were stored at −80°C for subsequent analysis. Body composition was analyzed by magnetic resonance imaging using an EchoMRI-100^{TM} analyzer (EchoMRI Medical Systems, Houston, TX, USA).

Run to exhaustion treadmill testing

Animals were acclimatized to running on an open treadmill (Columbus Instruments, Columbus, OH) for 10 min, 10 meters/min (m/min), 15° incline, on 2 consecutive days before the start of the run to exhaustion (RTE) tests. For the low intensity endurance RTE trial, mice started the run at 10 m/min for 10 min, and the speed was increased in 4 m/min increments every 10 min 22 m/min. After an hour at that speed, if mice were still running the speed was increased in 1 m/min increments every 5 min. Mice were motivated to run using bristled brushes at the back of the treadmill and were considered exhausted when they could no longer run in response to this stimulus. The experimenter was blinded to the genotype of the mice during the run tests. Blood

glucose was measured in tail vein blood before the start of the run and at exhaustion, using a handheld blood glucose meter.

Ex vivo 2-deoxy glucose uptake (2DOGU)

Ex vivo muscle insulin sensitivity was measured by the 2DOGU technique[53]. Mice were fasted for 4 hours before being anesthetized via intraperitoneal injection. Paired soleus and EDL muscles were incubated at 35°C for 30 minutes in oxygenated (95% O_2, 5% CO_2) flasks of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2mM Na-pyruvate, and 6mM mannitol. One muscle per pair was incubated in KHB without insulin, and the contralateral muscle was incubated in KHB with insulin (60 μU/ml [0.36 nM]). After 30 minutes, muscles were transferred to a second flask and incubated at 35°C for 20 minutes in KHB plus 0.1% BSA, 9 mM [^{14}C]-mannitol (0.053 mCi/mmol; PerkinElmer), and 1 mM [^{3}H]-2DG (6 mCi/mmol; PerkinElmer), with the same insulin concentration as in the first incubation. 2DOGU rate was calculated as previously described[53]. The soleus muscles used for 2DOGU measurements (i.e., 50 minutes without or with insulin stimulation) were used to measure phosphorylation of Akt (p-Akt^{Ser473}, p-Akt^{Thr308}), as well as phosphorylation of glycogen synthase kinase 3 beta GSK3β (p-GSK3β^{S79}).

Oral glucose tolerance test

Mice were fasted for 4 hours and then orally gavaged with 4 g of dextrose per kg body weight, and blood glucose was measured from tail vein blood at various time points; 0 (before gavage), 20, 30, 45, 60, 90 and 120 min; using a standard handheld glucose meter. Area under the curve (AUC) was calculated using Prism 7 (GraphPad Software Incorporated, La Jolla, CA, USA), and using time=0 as the baseline.

High-resolution respirometry

High-resolution respirometry was performed using an Oroboros O2K (Oroboros Instruments, Innsbruck, Austria). Briefly, excised TRI muscle preserved in biopsy preservation solution (BIOPS; 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.7 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM DTT, and 50 mM MES) was mechanically separated under a dissecting microscope and permeabilized with 50 μg/ml saponin for 20 min followed by two 15 min washes in MiR05 buffer [0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 110 mM sucrose, and 1 g/L fatty acid-free bovine serum albumin]. All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. All data were collected at 37°C in hyperoxygenated (200–450 μM) conditions in MiR05. There was 1 substrate-uncoupler-inhibitor titrations (SUITs) performed. The SUIT respiration protocol was the following: 0.5 mM malate, 0.2 mM octanoylcarnitine, 2.5 mM ADP, 10 μM cytochrome *c*, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate, and 1 μM carbonyl cyanide *m*-chloro phenyl hydrazone, followed by 0.5 μM rotenone and 2.5 μM antimycin a.

Complex IV/Citrate Synthase Activity Assays

Powdered GA (30–50 mg) was homogenized on ice with glass-on-glass homogenizing tubes in 0.5 ml ice-cold Zheng buffer [210 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1 mM EGTA (pH to 7.2 using potassium hydroxide)]. Sample homogenates were divided into aliquots and then underwent 3 freeze-thaw

cycles using a methanol/dry ice bath for enzyme analysis of complex IV. Homogenates were further disrupted by sonication for analysis of citrate synthase (CS). Enzyme activities were measured by spectrophotometric assays as previously described with minor modifications. All assays were performed in a 96-well plate using a Synergy HT spectrophotometer (BioTek, Winooski, VT, USA) at 30°C, in a final volume of 0.25 ml, in 50 mM potassium phosphate buffer (pH 7.4), unless otherwise indicated. For all assays, muscle homogenate was diluted with dH2O and protein concentration was optimized per enzyme to maximize the linearity of the reaction. Optimal protein concentrations per well were 10ug for CS and 20ug for CIV. Enzymatic activity was calculated using Beer's Law with the appropriate extinction coefficient: Activity (nmol*min –1 *mg –1) = (Δ Absorbance*min –1 *1,000)/[(Extinction coefficient * Volume of sample) × (Protein concentration)].

Immunoblotting

30 μg of protein per sample was separated by SDS-PAGE on XT Criterion Precast gels (Bio-Rad Laboratories, Hercules, CA, USA) under reducing conditions, transferred to Amersham Protran nitrocellulose membranes (MilliporeSigma, Burlington, MA, USA), and stained on the nitrocellulose membranes reversibly using a Ponceau S solution (0.1% [w/v] Ponceau S in 5% acetic acid). The membranes were blocked with 5% milk in TBST for 1 hour prior to an overnight incubation with the following primary antibodies; SIRT1 (3931, Cell signaling), GCN5L2 (3305, Cell signaling), SIRT3 (5490, Cell signaling), VDAC1 (4661, Cell signaling), PDHα (3205, Cell signaling), eukaryotic translation elongation factor 2 (eEF2; 2332, Cell signaling), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; D16H11, Cell signaling), ATP synthase subunit alpha (ATP5A), ubiquinol-cytochrome C reductase core protein 2 (UQCRC2), mitochondrially encoded

cytochrome C oxidase I (MTCO1), succinate dehydrogenase subunit B (SDHB), NADH:ubiquinone oxidoreductase subunit B8 (NDUFB8) (MS604, MitoSciences), acyl-Coenzyme A dehydrogenase, very long-chain (ACADVL; ab155138, Abcam), acyl-Coenzyme A dehydrogenase, long-chain (ACADL; ab82853, Abcam), hexokinase 2 (HK2; 2857, Cell signaling), lactate dehydrogenase A (LDHA; ABN896, MilliporeSigma), PKM 1/2 (3190, Cell signaling), Akt (2920, Cell signaling), pAkt S473 (4058, Cell signaling), pAkt T308 (9275, Cell signaling), GSK3α/β (5676, Cell signaling), pGSK3α/β S21/S9 (9331, Cell signaling), pACC S79 (3661, Cell signaling), ACC (3676, Cell signaling) pAMPKa Thr172 (2535, Cell signaling), AMPKa (2793, Cell signaling). Densitometric analysis of immunoblots was performed on four or seven individual samples using Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA), and a representative image is presented in each figure.

RNA extraction and RT-PCR

Equal amounts of RNA (1 ug) were used for cDNA synthesis after being extracted from snap-frozen gastrocnemius muscle using TRIzol Reagent (ThermoFisher Scientific, Waltham, MA, USA). Semiquantitative real-time PCR analysis was performed using iTaq SYBR Green master mix (Bio-Rad) on a CFX384 touch real-time PCR system (Bio-Rad). The ΔΔCt method was used to calculate relative levels of gene expression; *Eef2* or *PolR2A* was used as a normalization control. The sequences for primers used in this study can be found in Table 2.

Statistics

Data were analyzed using an unpaired Student's t-test or 2-way ANOVA (using repeated measurement where appropriate), followed by a Tukey's post-hoc test, with significant differences

at $p<0.05$. Statistical analyses were performed using Prism 7 (GraphPad Software Incorporated, La Jolla, CA, USA). All data are expressed as mean ±SEM.

RESULTS

Overexpression of SIRT1 and knockout of GCN5 in adult mouse skeletal muscle

To confirm that SIRT1 was overexpressed and GCN5 was knocked out in adult skeletal muscle of dTG mice, we first assessed changes in SIRT1 and GCN5 gene expression and protein abundance. Sirt1 gene expression was increased by 390% and Gcn5 gene expression was reduced by 70% in skeletal muscle of dTG mice vs. WT mice (Figure 1A), while Pgc1α and Pcaf transcript abundance was unchanged (Figure 1A). Altered gene expression of SIRT1 and GCN5 was associated with increased SIRT1 protein abundance and a significant reduction in GCN5 protein abundance in dTG vs. WT mice (Figure 1B-C).

dTG mice show no differences in tissue weights or body composition

There were no differences in body weight, or weights of heart, liver, epididymal adipose tissue, skeletal muscle (GA, TA, or the Quad) (Table 1), or body composition between dTG and WT mice (Figure 1D).

dTG mice show increased gene expression of mitochondrial proteins

We next assessed transcriptional changes in mitochondrial proteins in the muscle of dTG and WT mice. Gene expression of mitochondrial proteins such as cytochrome c oxidase subunit 5B (Cox5b), citrate synthase (Cs), cytochrome C (Cycs), succinate dehydrogenase complex iron sulfur subunit b (Sdhb), and Sirtuin 3 (Sirt3) were increased, while transcript levels of nuclear respiratory factor 2 (Nrf2) were unchanged (Figure 2A). In conjunction with the increase in transcript abundance, SIRT3 protein abundance was also increased (Figure 2B-C). There was an approximately 25% increase in the abundance of electron transport chain protein ATP synthase subunit alpha (ATP5A), however there was no increase in the abundance of ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), cytochrome c oxidase subunit I (MTCO1),

SDHB, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8), or voltage-dependent anion-selective channel 1 (VDAC1) when comparing dTG vs WT mice. (Figure 2B-C). There was a minor but significant increase in citrate synthase activity in skeletal muscle when comparing dTG to WT mice, however there were no changes in the activity of Complex IV of the ETC (Figure 2D-E).

Markers of fatty acid, but not carbohydrate, oxidation are increased in dTG skeletal muscle

To investigate changes in fatty acid and carbohydrate metabolism, we measured metabolic transcripts/proteins in the gastrocnemius. mRNA expression of acyl-CoA dehydrogenase long chain (Acadl) and acyl-CoA dehydrogenase very long chain (Acadvl) were both increased in dTG muscle compared to WT (Figure 3A). Despite the increase in gene expression, the protein abundance of ACADL was not significantly increased (Figure 3A-C). There were no differences between genotypes in gene expression or protein abundance related to glucose metabolism (e.g. HK2, PKM1/2, PDHα, LDHA, and GLUT4), as well as no observed increases in phosphorylation levels of Acetyl-CoA carboxylase (ACC) or adenosine monophosphate-activated protein kinase (AMPK), proteins that are important in modulating fat and carbohydrate oxidation (Figure 3 D-H).

dTG skeletal muscle does not have increased maximum respiratory capacity

To investigate if changes at the gene and protein level translated into functional improvements in maximal respiration of skeletal muscle, we assessed mitochondrial function in permeabilized plantaris fiber bundles using high resolution respirometry. Interestingly, we found no differences between genotypes in maximal respiratory capacity, ETC capacity, leak respiration

(absence of adenylates), residual oxygen consumption, and oxidative phosphorylation-coupling efficiency between genotypes (Figure 4A). We found no differences between genotypes in the flux control ratios for any of the specific complexes, defined as, $\frac{Flux\ throu\quad complex}{Maximum\ uncoupled\ flux}$ (Figure 4B). We also assessed changed in exercise capacity during an acute low-intensity exercise bout and found no differences between genotype for time spent running, distance run, or blood glucose basally or at exhaustion (Figure 4C-E).

Skeletal muscle insulin sensitivity is comparable in dTG and WT mice

Lastly, we checked for functional changes in glucose homeostasis. While there was no significant difference in blood glucose concentration at any time point during the OGTT except for at 120 min, there was a significant main effect for genotype. In line with this, the glucose AUC during the GTT trended to be higher (P=0.073 in dTG versus WT by 26% (Figure 5A). Given our model is skeletal muscle-specific, we next determined whether skeletal muscle insulin sensitivity was changed using an ex vivo, 2DOGU approach. While 2DOGU in the presence of insulin was significantly higher than basal 2DOGU in both soleus and EDL, there were no genotype differences (Figures 5C-D). Moreover, insulin-stimulated 2DOGU (i.e. Insulin 2DOGU minus Basal 2DOGU) there was not different between genotypes in either muscle (Figure 5E). In line with the 2DOGU findings, there were no genotype differences in Akt T308 or S473 phosphorylation, although there was a significant effect of insulin (Figure F-G).

DISCUSSION

The individual contributions of SIRT1 and GCN5 activity to metabolism and mitochondrial function through control of PGC1α activity have been well studied, however the interplay of these enzymes in skeletal muscle has not yet been investigated. The purpose of this study was to investigate the roles of SIRT1 and GCN5 in skeletal muscle metabolic remodeling and mitochondrial biogenesis. To that end, we generated a mouse model with inducible, skeletal muscle-specific SIRT1 overexpression and GCN5 knockout. The major finding of this study was that despite successful overexpression of SIRT1 and knockout of GCN5 knockout in skeletal muscle, there were no functional changes in mitochondrial function, running capacity or skeletal muscle insulin sensitivity.

SIRT1 is a potent activator of PGC1α(Rodgers et al. 2005; Nemoto, Fergusson, and Finkel 2005) and as such has been identified as a regulator of mitochondrial biogenesis(Philp and Schenk 2013; Yuan et al. 2016). For example, whole-body overexpression of SIRT1 in mice increased PGC1α expression when tested for in skeletal muscle along with other mitochondrial genes and state 3 mitochondrial respiration(Dominy et al. 2010). Muscle-specific overexpression of SIRT1 increased the abundance of some electron transport chain proteins(Banks et al. 2008; Amanda T. White et al. 2014). In line with these observations, activation of SIRT1 through elevated cellular NAD^+ levels increases mitochondrial biogenesis in skeletal muscle(Bai, Cantó, et al. 2011; Cantó et al. 2012; Bai, Canto, et al. 2011). Overexpression of GCN5 in C2C12 myotubes resulted in repression of PGC1α-mediated induction of mitochondrial genes(Gerhart-Hines et al. 2007), and in HEK293 cells also lead to repression of PGC1α's transcriptional activity(Lerin et al. 2006). Knockout of GCN5 in the liver lead to an increase in the transcript abundance of PGC1α and an

increase in mitochondrial ROS production(Lingdi Wang et al. 2017). Based on these studies, we hypothesized that our dTG mouse model would exhibit enhanced mitochondrial function and abundance in skeletal muscle due to an additive effect of overexpressing SIRT1 and knocking out GCN5. To our surprise, dTG skeletal muscle did not show changes in either of their parameters.

Although our results were surprising, they are supported by other studies that have been done in the field; SIRT1 activity was not positively correlated with enhancements in oxidative capacity in rat skeletal muscle, and overexpression in the skeletal muscle led to decreased mitochondrial biogenesis(Gurd et al. 2009). In line with these observations, temporal overexpression of SIRT1 in rodent skeletal muscle did not improve mitochondrial biogenesis(Brandon et al. 2015; K. Svensson et al. 2017). Muscle-specific knockout of GCN5 did not lead to changes in mitochondrial density or maximum respiratory capacity in permeabilized muscle fibers(Dent et al. 2017). The increases in the transcript abundance of mitochondrial genes led us to conclude that PGC1α activation was in fact increased. However, this alone was not sufficient to induce changes in mitochondrial function or protein abundance, leading us to conclude that there may be another "signal" necessary to induce mitochondrial changes. For instance, AMPK was shown to phosphorylate PGC1α at threonine 177 and serine 538 leading to increased transcription of mitochondrial genes(Jäger et al. 2007). This may also help to explain the mitochondrial adaptations seen in skeletal muscle after pharmacological activation of SIRT1 via resveratrol(Menzies et al. 2013; Timmers et al. 2011), as it has been shown that resveratrol also activates AMPK in skeletal muscle(Higashida et al. 2013). It was also seen that SRT1720 required activation of AMPK for improvements in glycemic control in mice(Park et al. 2017). In all, results from this current study, past results from our laboratory(Dent et al. 2017; K. Svensson et al. 2017) as well as others(Gurd et al. 2009; Brandon et al. 2015) show that changes in SIRT1

and/or GCN5 activity alone are not sufficient to induce functional mitochondrial changes or changes in mitochondrial density in skeletal muscle, in vivo.

In primary skeletal muscle myotubes, expression of GCN5 lead to a decrease in insulin mediated glucose uptake(Kelly et al. 2009), and whole-body overexpression of GCN5 in mice decreased hepatic glucose production(Lerin et al. 2006). In line with these observations, mice with a muscle-specific knockout of SIRT1 did not experience improvements in insulin sensitivity brought on by caloric restriction (CR)(Schenk et al. 2011). SIRT1 whole body overexpression also exhibits a protective effect against insulin resistance brought on by a high fat diet (HFD)(Paul T Pfluger et al. 2008; Banks et al. 2008). Overexpression of SIRT1 in the offspring of mice that were fed a maternal HFD were also protected from decreased glucose tolerance and insulin sensitivity brought on by the maternal HFD(Nguyen et al. 2018). Pharmacological activation of SIRT1 in obese rodents and humans also leads to improved glucose tolerance as well as improved insulin sensitivity(Lagouge et al. 2006; Feige et al. 2008). Our dTG mice did not show any changes to insulin sensitivity and only very minor changes in glucose tolerance, leading us to believe that while SIRT1 and GCN5 function are important in glucose homeostasis, it is not their activity in skeletal muscle that leads to these beneficial changes in vivo and more work needs to be done to elucidate exactly where these enzymes exhibit their effects.

The beneficial metabolic effects of increased SIRT1 activity and decreased GCN5 activity have been well documented, however the specific organ responsible for these benefits has yet to be determined. SIRT1 has been shown to be highly expressed in the brain, liver, and pancreas as well, other organs that are important for glucose homeostasis in the body(Haigis and Sinclair 2010). When SIRT1 is overexpressed in adipose tissue and the brain, but not the liver or skeletal muscle, glucose tolerance is improved(Bordone et al. 2007). Mice with germline muscle-specific

overexpression of SIRT1 as well as mice with inducible muscle-specific overexpression of SIRT1 did not show changes in insulin sensitivity(A. T. White et al. 2013; K. Svensson et al. 2017), muscle-specific overexpression of SIRT1 did not prevent impairments in insulin sensitivity brought on by a HFD(Amanda T. White et al. 2014), and lastly a muscle-specific GCN5 knockout did not induce any changes in in vivo metabolism or energy expenditure(Dent et al. 2017); supporting our claim that skeletal muscle SIRT1 or GCN5 activity is not responsible for the beneficial metabolic effects observed in other models.

In conclusion, we demonstrated that temporal overexpression of SIRT1 with concurrent knockout of GCN5 specifically in adult mouse skeletal muscle does not lead to enhanced mitochondrial biogenesis, functional changes to mitochondria, enhanced exercise capacity, improved insulin sensitivity, and only slightly enhances glucose tolerance despite enhanced gene expression of mitochondrial proteins and those involved in fatty acid oxidation. Our results support previous studies that found no effect of individual manipulation of skeletal muscle SIRT1 activity or GCN5 activity on in vivo metabolic and mitochondrial adaptations(Amanda T. White et al. 2014; A. T. White et al. 2013; Dent et al. 2017; K. Svensson et al. 2017). It would be a benefit to investigate the interplay of these two enzymes on metabolism in other organs, as well as if an outside stimulus, such as exercise training, could act as a "signal" to induce further mitochondrial adaptation in dTG mice compared to WT.

This thesis, in whole, is currently being prepared for submission for publication of the material. Tahvilian, Shahriar; Svensson, Kristoffer; Martins, Vitor F.; Dent, Jessica R.; Greyslak, Keenan; McCurdy, Carrie E.; Schenk, Simon. The thesis author was the primary investigator and author of this material.

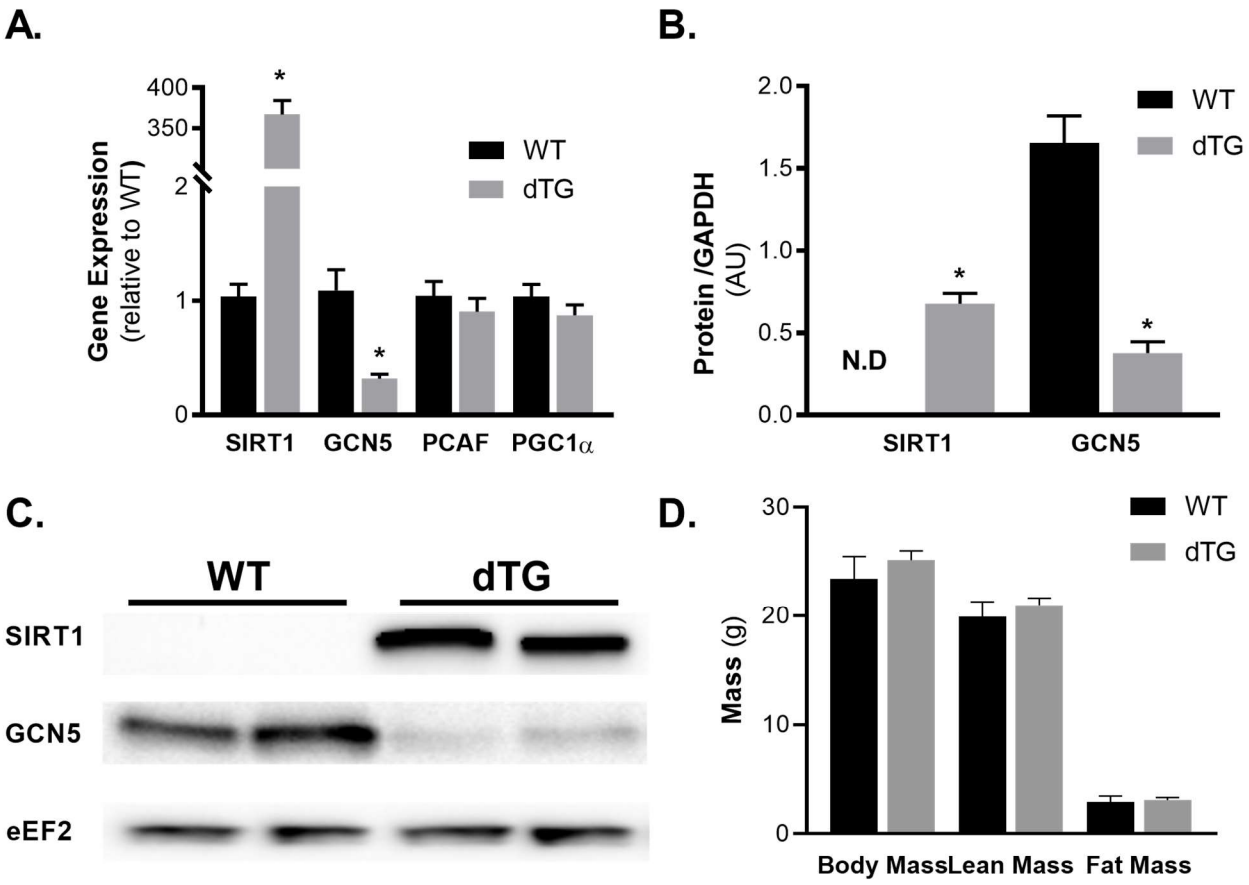
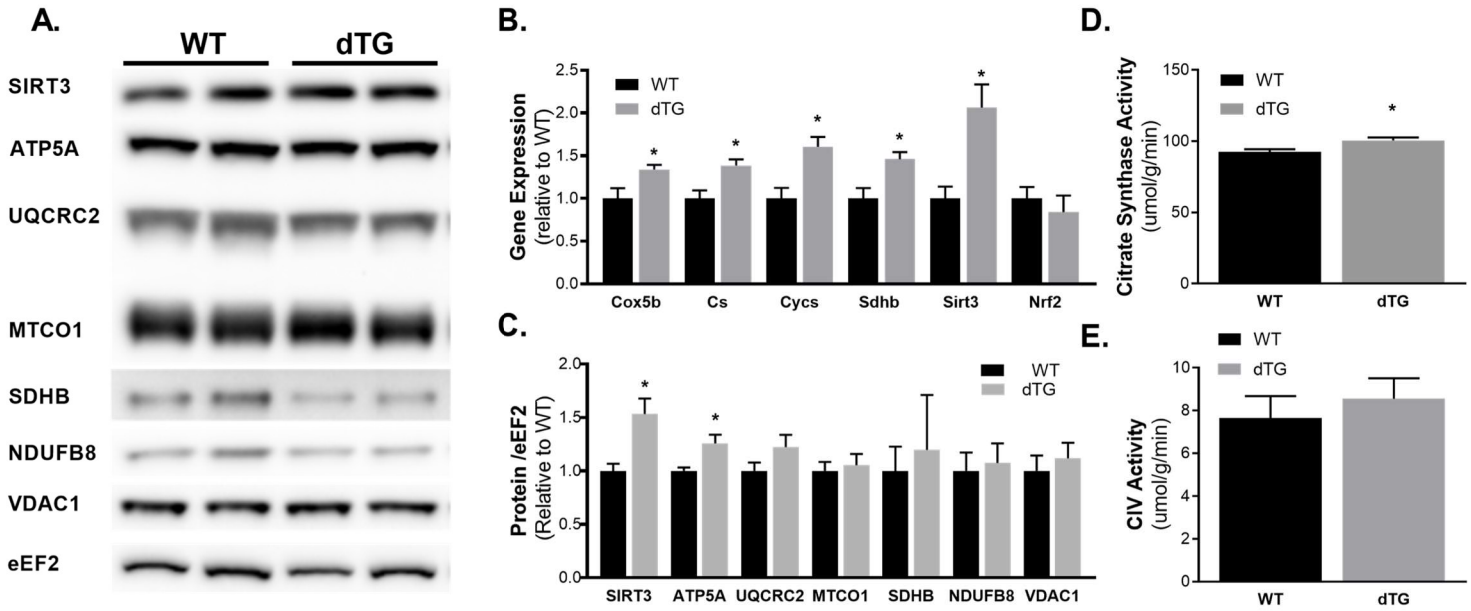


Figure 1: Mice with inducible muscle specific overexpression of SIRT1 and knockout of GCN5 (dTG) show increased levels of SIRT1 and decreased levels of GCN5 in skeletal muscle. (A) Transcript levels of Sirt1, Gcn5, Pcaf, and Pgc1α from WT and dTG skeletal muscle. n = 7 for all groups. (B) Representative blot of SIRT1 and GCN5 in skeletal muscle from WT and dTG. (C) Quantification of protein abundance in skeletal muscle whole cell lysate. n = 7 for all groups. (D) Body weight, lean mass, and fat mass of WT and dTG mice. WT (n = 5), dTG (n = 7). Data reported as mean +/- SEM. *$p < 0.05$ when compared to WT.

Table 1: Tissue weights of WT and dTG mice. WT (n = 9), dTG (n = 8). Data reported as mean +/- SEM.

Tissue	WT	dTG
BW (g)	25.34 ± 1.21	25.23 ± 1.11
GA (mg)	110.2 ± 5.9	100.5 ± 5.2
TA (mg)	48.4 ± 1.5	46.5 ± 1.8
QUAD (mg)	168.4 ± 8.6	167.1 ± 9.7
Heart (mg)	124.1 ± 4.5	112.2 ± 5.7
Liver (mg)	1341.8 ± 88.5	1316.6 ± 99.0
eWAT (mg)	428.0 ± 48.5	441.5 ± 93.0

Figure 2: Overexpressing SIRT1 and knocking out GCN5 in skeletal muscle leads to enhanced gene expression of mitochondrial proteins. (A) Representative blot of electron transport chain proteins (ATP5A, UQCRC2, MTCO1, SDHB, NDUFB8), as well as other mitochondrial proteins (SIRT3, VDAC1). (B) Transcript levels of mitochondrial proteins (Cox5b, Cs, Cycs, Sdhb, Sirt3) from WT and dTG skeletal muscle. n = 7. (C) Quantification of protein abundance in skeletal muscle whole cell lysate. n = 7 for all groups. (D) Citrate synthase activity is increased in dTG skeletal muscle. (E) Complex IV activity is unchanged in dTG skeletal muscle. n = 7 for all groups. Data reported as mean +/- SEM. *$p < 0.05$ when compared to WT.


A.
WT
dTG
SIRT3
ATP5A
UQCRC2
MTCO1
SDHB
NDUFB8
VDAC1
eEF2
B.
Gene Expression
(relative to WT)
WT
dTG
Cox5b
Cs
Cycs
Sdhb
Sirt3
Nrf2
C.
Protein /eEF2
(Relative to WT)
SIRT3
ATP5A
UQCRC2
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Citrate Synthase Activity
(umol/g/min)
E.
CIV Activity
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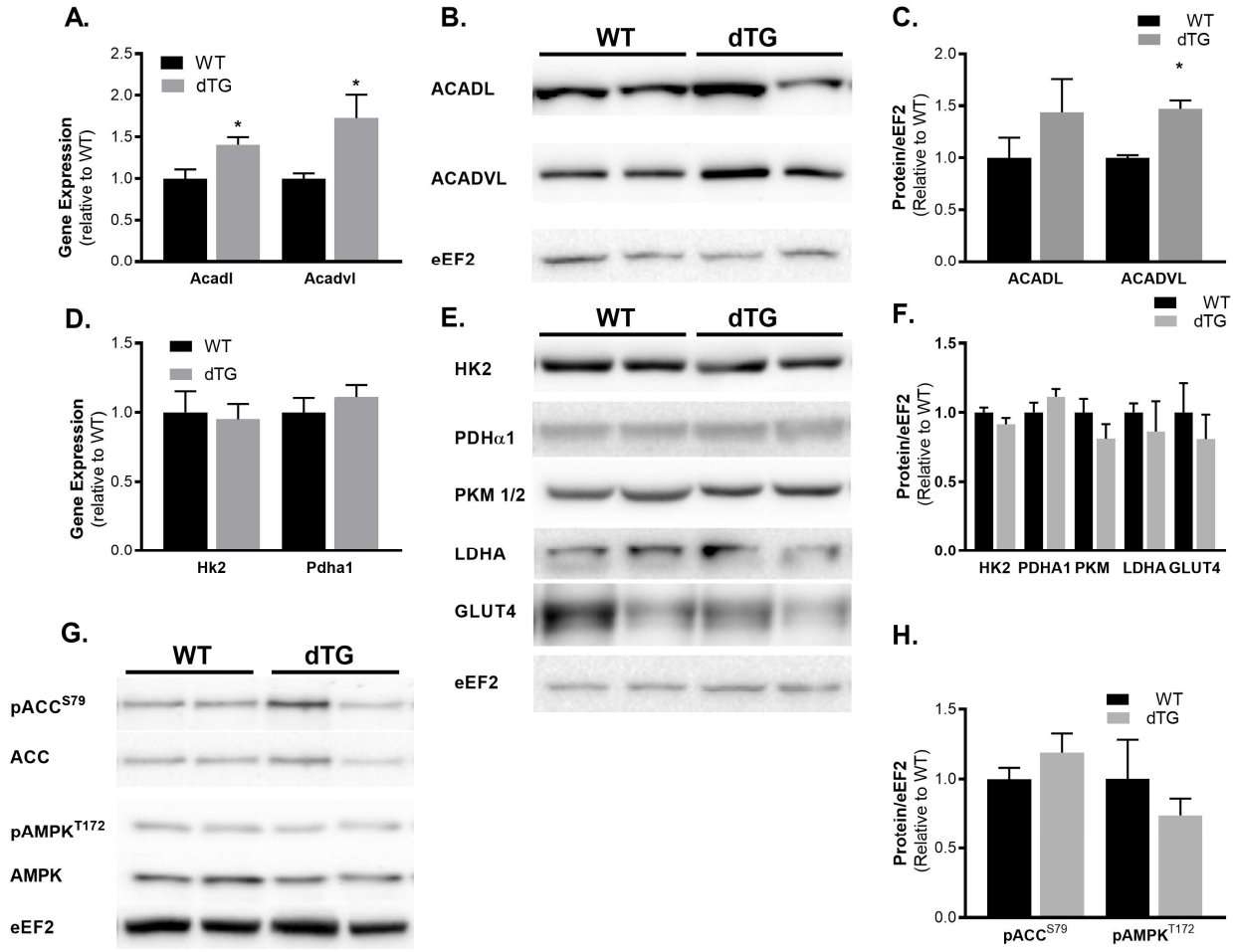
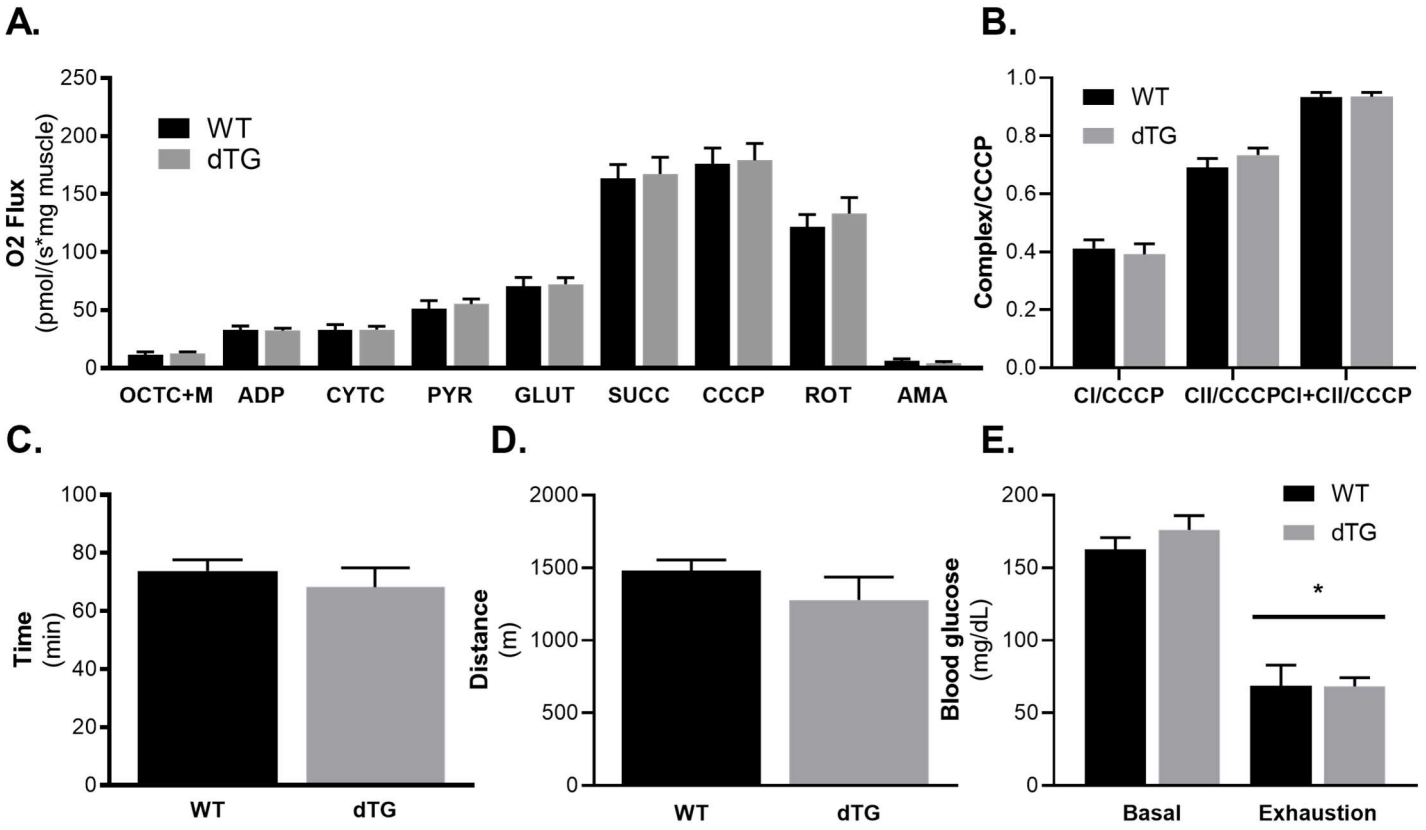


Figure 3: dTG mice have increased expression of fatty acid oxidation genes. (A) Transcript levels of fatty acid oxidation genes (Acadl and Acadvl). n = 7 for all groups. (B) Representative blot of fatty acid oxidation genes in WT and dTG mice. (C) Quantification of protein abundance in whole cell lysate. n = 7 for all groups. (D) Transcript levels of proteins involved in the metabolism of glucose (Hk2, Pdha1). (E) Representative blot of proteins involved in the metabolism of glucose (HK2, PKM, PDHa1, LDHA) in WT and dTG skeletal muscle. (F) Quantification of protein abundance in skeletal muscle whole cell lysate. (G) Representative blot of total and phosphorylated ACC, as well as total and phosphorylated AMPK in WT and dTG skeletal muscle. (H) Quantification of protein abundance in skeletal muscle whole cell lysate. n=7 for all groups. Data reported as mean +/- SEM. *p < 0.05 when compared to WT.

Figure 4: dTG mice show no changes in mitochondrial function when compared to WT. (A) Respiratory flux normalized to muscle fiber weight in the presence of MOct (leak respiration in the absence of adenylates), ADP (D), cytochrome c (C; mitochondrial integrity), pyruvate (P), glutamate (G; complex I (CI) capacity), succinate (S; complex I + complex II (CII) capacity), carbonyl cyanide m-chloro phenyl hydrazone (U; maximal respiration), rotenone (Rot; complex II capacity), and Ama (residual oxygen consumption (Rox)). (B) Flux control ratios calculated as $\frac{Complex\ Capacity}{Uncoupled\ Maximum\ flux}$. WT (n = 9) dTG (n = 7). (C) Run to exhaustion time and (D) distance recorded during an acute exercise bout. (E) Blood glucose concentration before and after exhaustion during an acute exercise bout. n = 6 for all groups. Data reported as mean +/- SEM. *$p < 0.05$ effect of exhaustion.


A.
O2 Flux
(pmol/(s*mg muscle)
250
200
150
100
50
0
WT
dTG
OCTC+M
ADP
CYTC
PYR
GLUT
SUCC
CCCP
ROT
AMA
B.
Complex/CCCP
1.0
0.8
0.6
0.4
0.2
0.0
WT
dTG
CI/CCCP
CII/CCCP
CI+CII/CCCP
C.
Time
(min)
100
80
60
40
20
0
WT
dTG
D.
Distance
(m)
2000
1500
1000
500
0
WT
dTG
E.
Blood glucose
(mg/dL)
200
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100
50
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WT
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Basal
Exhaustion

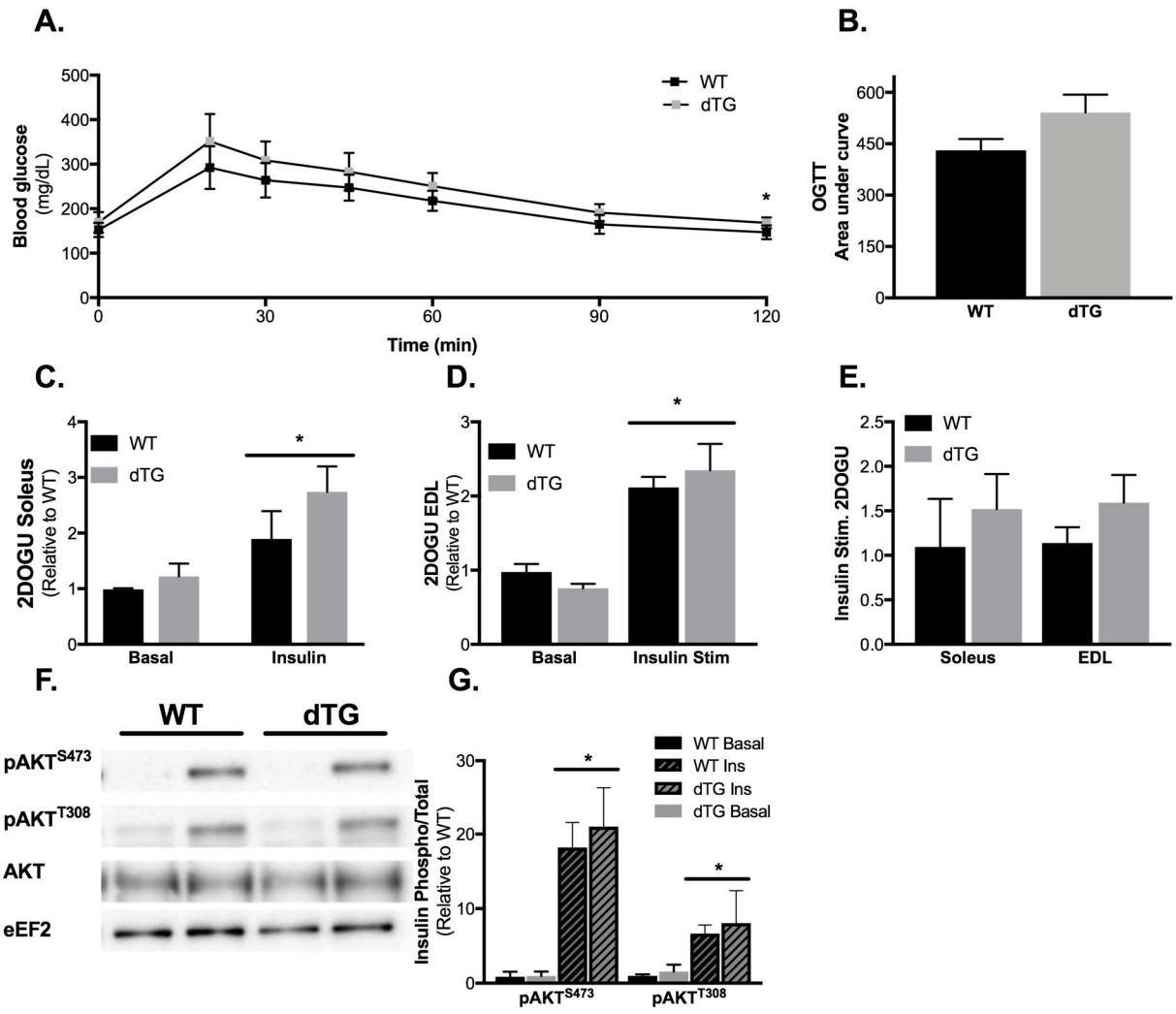

Figure 5: dTG mice show no changes in glucose homeostasis when compared to WT. (A) Blood glucose concentrations (*p<0.05 when compared to WT) and (B) area under the curve (AUC) of WT and dTG mice during an oral glucose tolerance test (OGTT) during which mice were given 4g dextrose per kg body weight via oral gavage. WT (n = 14), dTG (n = 8) (C-E) Insulin stimulated (0.36 nmol/L) 2-deoxy-glucose uptake (2DOGU) in isolated soleus and EDL muscles from WT and dTG mice. WT (n = 6), dTG (n = 8). Basal and insulin 2DOGU in (C) soleus and (D) EDL muscles from WT and dTG mice. (E) Insulin-stimulated (Insulin Stim.) 2DOGU (calculated as insulin 2DOGU – basal 2DOGU) in soleus and EDL muscle from WT and dTG mice. (F) Phospho-Akt^{S473} (pAkt^{S473}), phospho-Akt^{T308} (pAkt^{T308}), total Akt, phospho-glycogen synthase kinase 3 beta^{S9} (pGSK3β^{S9}), total glycogen synthase kinase 3 beta (GSK3β) in basal (B) and insulin stimulated (I) EDL muscle from WT and dTG mice. WT (n = 5) dTG (n = 3). (G) Quantification of pAkt^{S473}, pAkt^{T308}, and pGSK3β^{S9} compared to their respective total protein in basal and insulin-stimulated EDL muscle from WT and dTG mice. WT (n = 5) dTG (n = 3). Data reported as mean +/- SEM. *p<0.05 effect of insulin.

Table 2: qPCR primer sequences.

Primer	Forward	Reverse
Sirt1	GGCCTAATAGACTTGCAAAGGA	CTCAGCACCGTGGAATATGTAA
Gcn5	CAGGTCAAGGGCTATGGCAC	GATAGCGGCTCTTGGGCAC
Pcaf	AGAAGAAGCCGCCATTTGAGA	CGATCGTTGTCTGCCTCTCTT
Pgc1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Cox5b	AAGGGACTGGACCCATACA	ACAGATGCAGCCCACTATTC
Cs	TCCTGGTCGTTTGGCTTTATC	GTTCCGTGCCAGAGCATATT
Cycs	GAGGATACCCTGATGGAGTATTTG	GCTATTAGGTCTGCCCTTTCTC
Sdhb	CTGCCACACCATCATGAACT	CTTGTAGGTCGCCATCATCTTC
Sirt3	GTTCTGAGTCCTCGAAGGAAAG	AGATCCAGCAGTTCTTGTGTC
Nrf2	ACAGAGGCATTTTATAGCCATGTG	TGCTTCTGCCTCCTGAATGTC
Acadvl	CTTTGCAGGGACTCAAGGAA	CAAGCGAGCATACTGGGTATTA
Acadl	CTCAGGACACAGCAGAACTATT	GCTCTTGCATGAGGTAGTAGAA
Hk2	GCTGGAGGTTAAGAGAAGGATG	TGGAGTGGCACACACATAAG
Pdha1	AGAGAGGATGGGCTCAAGTA	CAAGTGACAGAAACCACGAATG

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