Identifying the enzymes that regulate acetylation of sarco(endo)plasmic reticulum calcium ATPase 2a (SERCA2a)

by:

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Abstract

The Sarco(endo)plasmic Reticulum Calcium ATPase 2a (SERCA2a) is the primary cardiomyocyte calcium transporter and has been shown to be acetylated at higher levels in the diabetic mouse heart. The objective of this study was to determine if SERCA2a acetylation changes with high glucose exposure, which enzyme(s) deacetylate SERCA2a, and which enzyme(s) acetylate SERCA2a. We hypothesized that increased SERCA2a acetylation will have a negative effect on SERCA2a function and that the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate SERCA2a acetylation. Our data demonstrates that high glucose increases SERCA2a acetylation and leads to decreased SERCA2a activity in primary rat cardiomyocytes. SERCA2a acetylation increased with inhibition of the sirtuins but Class I & II HDACs. SERCA2a acetylation decreased with pan-inhibition of histone acetyltransferases (HATs) and inhibition of GCN5 prior to glucose exposure. Together this data identifies the sirtuins and GCN5 as possible therapeutic targets to decrease SERCA2a acetylation.

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List of Abbreviations

AAV – Adeno-associated virus

Acetyl-CoA – Acetyl co-enzyme A

AMP – Adenosine monophosphate

AMPK – AMP-activated protein kinase

ATP – Adenosine triphosphate

ANOVA – Analysis of variance

BSA – Bovine serum albumin

Ca^{2+} – Calcium

Ca_{50} – Calcium concentration needed to elicit ½ V_{max} (Calcium Sensitivity)

CaMK – Ca^{2+}/calmodulin-dependent protein kinase

CaMKII – Ca^{2+}/calmodulin-dependent protein kinase II

CBP – CREB-binding protein

CPA – Cyclopiazonic acid

CUPID – Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease

GCN5 – General control non-depressible 5

GCN5L1 – GCN5-like protein 1

GLUT – Glucose transporter

FOXO – Forkhead box O

HAT – Histone acetyltransferase

HbA1C – Glycated Hemoglobin

HDAC – Histone deacetylase

K_m – Michaelis Constant

LKB1 – Liver Kinase B1

LV – Left ventricle

LVAD – Left ventricular assist device

NYHA – New York Heart Association

V_{max} – Maximal Rate

p300 – E1A binding protein p300

PBS – Phosphate buffered saline

PCr – Phosphocreatine

PGC1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PKA – Protein kinase A (cyclic-AMP-dependent protein kinase)

PLN – Phospholamban

PVDF – Polyvinylidene fluoride

ROS – Reactive oxygen species

RyR – Ryanodine receptor

SERCA – Sarco(endo)plasmic reticulum Ca^{2+}-ATPase

Sir2 – Silent information regulator 2

SIRT1 – Sirtuin 1

SIRT2 – Sirtuin 2

SIRT3 – Sirtuin 3

SLN – Sarcolipin

Small ubiquitin-like modifier 1 – SUMO1

SR – Sarcoplasmic reticulum

TBST – Tris-buffered saline tween

TFAM – Mitochondrial transcription factor A

TFB2M – Mitochondrial transcription factor B2

TIP60 – 60 kDa Tat-Interactive Protein

TSA – Trichostatin A

WT – Wild-type

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Chapter 1: Literature Review

Diabetes results in significant functional changes within the heart

Currently, there are 2 million Canadians living with diabetes and the incidence of diabetes continues to rise [1]. In conjunction with this, the number of patients having complications from diabetes will continue to rise accordingly. The most common cause of medical complications and death in diabetics is cardiovascular disease [2]. Diabetic patients frequently suffer from a diabetic cardiomyopathy, which is defined as pathologic changes in the structure and function of the heart which cannot be directly attributed to other factors such as coronary artery disease or hypertension [3]. There are several pathophysiological changes that occur in the diabetic heart that appear to act synergistically to impair cardiac function. Hyperglycemia is considered to be a key trigger of this decline in cardiac function, due to its ability to trigger several of the maladaptive changes that occur in the diabetic heart [3]. Aggressive glycemic control has been shown to reduce microvascular complications, with a 1% reduction in glycated hemoglobin (HbA1C) leading to a 37% reduction in microvascular complications [4]. The benefits are less clear in regard to macrovascular complications. In older patients who are at risk for, or have established cardiovascular disease, there was no significant benefit to aggressive glycemic control [6]. Conversely, in newly diagnosed patients with a low HbA1C and no evidence of macrovascular disease, there appears to be a long lasting benefit to aggressive glycemic control [5].

Fatty acids are the primary energy source for the heart [6]. In a non-diabetic state, the heart is able to easily adapt and alter substrate utilization as needed [7]. For example, in

chronic ischemic conditions the heart is able to utilize a larger proportion of glucose for energy as glycolysis generates less oxygen demand than fatty acid oxidation [7]. However, when diabetes is present, there is an increase in fatty acid oxidation and a decrease in glucose utilization as a result of reduced levels of glucose transporter (GLUT) -1 and -4 proteins, resulting in metabolic inflexibility [8]. The complete mechanism as to why GLUT-1 and GLUT-4 decrease in diabetes is not yet clear. However, the impairment of insulin signaling can partially account for this effect as these are, at least in part, insulin-dependent transporters [9]. Increased fatty acid oxidation requires a large amount of oxygen to produce adenosine triphosphate (ATP), resulting in increased cardiac oxygen demand and increased levels of toxic intermediates from fatty acid metabolism, which is called lipotoxicity [3]. Additionally, oxidative stress has also been suggested to play a role in the diabetes-induced cardiac impairment. These increased levels of reactive oxygen species (ROS) appear to originate from mitochondria as a result of increased fatty acid oxidation. Higher levels of ROS appear to lead to mitochondrial uncoupling, further impairing energetics in the myocardium [10]. Compromised energetics appear to be causally linked to the pathogenesis of heart failure and a low phosphocreatine (PCr) to ATP ratio has been correlated to disease severity and poor prognosis in heart failure patients [11–13]. Pinz *et. al* [14] suggest that this impairment of cellular energetics may play a role in a hypertrophied heart by limiting energetic support to SERCA2a. The renin-angiotensin system also plays a role in diabetic cardiomyopathy, as Angiotensin II receptor density is increased in the diabetic heart [15]. Furthermore activation of the renin-angiotensin system has been shown to increase oxidative stress and both apoptosis and necrosis of human cardiomyocytes [16].

To examine the effects of diabetes, several cell culture models have been developed. Although it doesn't fully mimic the pathologic milieu of diabetes, one commonly used approach is to introduce 25 mM glucose media to isolated cardiomyocytes to mimic elevated glucose levels found in diabetics [17,18]. Alternative approaches in cell culture include treatments with 100 nM insulin [19] or 200 μM palmitate [19,20]. These models result in similar contractile dysfunction to that experienced in humans with diabetic cardiomyopathy [21].

The importance of calcium in muscle contraction

Calcium (Ca^{2+}) plays a crucial role in regulating the process of excitation contraction coupling. In cardiac muscle, contraction is initiated when a depolarizing stimulus causes L-type Ca^{2+} channels to open within the T-tubules. The opening of the L-type Ca^{2+} channels results in increased entry of Ca^{2+} into the cell [22]. This Ca^{2+} interacts with the ryanodine receptors (RyR), which, in turn, release more intracellular Ca^{2+} from the sarcoplasmic reticulum. This process is called calcium-induced calcium release [23]. The concentration of free intracellular Ca^{2+} increases by approximately tenfold during this process [24]. This increase facilitates the binding of Ca^{2+} to the troponin complex causing a conformational change, which reveals the actin-myosin binding sites on the actin filament [25]. This conformational change allows myosin to interact with this binding site, resulting in an attachment between actin and myosin. The attachment of myosin to actin is coupled with ATP hydrolysis and a "power stroke" is generated. This power stroke movement is the process responsible for the contraction of the muscle [26].

To relax, intracellular Ca^{2+} must be removed and restored to basal levels. Four transporters contribute to Ca^{2+}removal: (1) the sarcolemmal Ca^{2+} ATPase; (2)

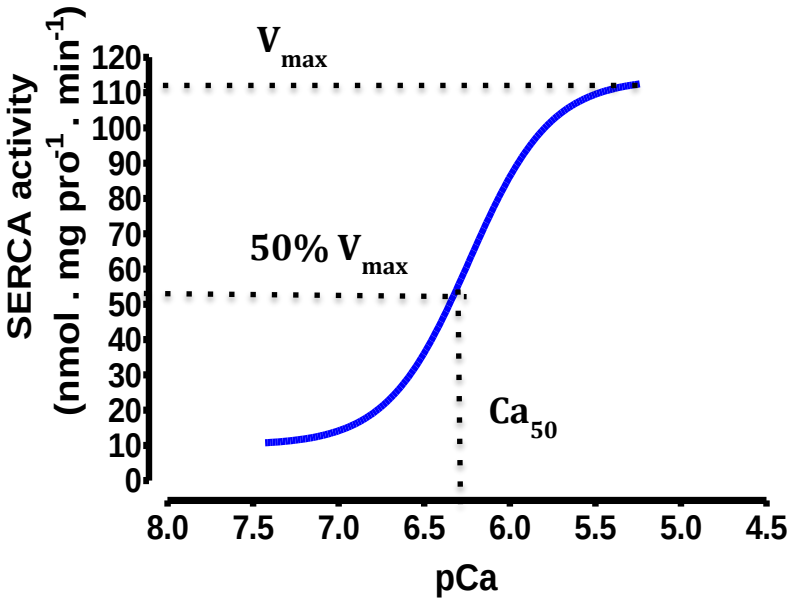
mitochondrial Ca^{2+} uniporter; (3) the Na^{+}/Ca^{2+} exchanger; and (4) the Sarco(endo)plasmic Reticulum Calcium ATPase (SERCA) [24]. Of these four transporters, SERCA is responsible for moving the predominant proportion of Ca^{2+} back into the sarcoplasmic reticulum in mammals and replenishing calcium stores for subsequent contractions [22]. In rodents, 92% of cytosolic Ca^{2+} is removed by SERCA2a; [22] whereas in the human heart, SERCA2a removes approximately 70% of cytosolic Ca^{2+} [27].

Structure and function of SERCA

SERCA belongs to the P-type ATPase family, a group of proteins that couple ATP hydrolysis to ion transport [28]. Structurally, SERCA is a transmembrane protein with a molecular mass of 110 kDa consisting of three distinct regions: the cytoplasmic head, transmembrane helices, and luminal loops [29]. The cytoplasmic head of SERCA is present on all SERCA isoforms and contains an actuator domain, phosphorylation domain, and nucleotide binding domain. Each of these three domains has a unique role in the function of SERCA. The nucleotide domain binds to adenosine and the phosphorylation domain binds to the γ-phosphate of the ATP molecule. Together, these two domains make up the catalytic site of SERCA [30]. When Ca^{2+} binds to the actuator site, it produces an important conformational change. Without the bound Ca^{2+}, the ATP γ-phosphate is unable to interact with the SERCA phosphorylation domain [31]. Binding of Ca^{2+} to the two active sites in the SERCA actuator domain occurs in a positive cooperative manner, where the binding of the first Ca^{2+} increases the affinity of the second site for Ca^{2+} [28,31]. Thus, SERCA transports 2 Ca^{2+} ions for every 1 ATP hydrolyzed [28].

SERCA exists as several isoforms that are expressed in different tissues. In muscle, the predominant isoform of SERCA present is determined by fiber type. SERCA1a is found in adult skeletal muscle while the SERCA1b isoform is found in neonatal skeletal muscle [32]. SERCA2a and SERCA2b are found in both slow twitch and cardiac muscle; however, SERCA2a is significantly more abundant in these tissues, as SERCA2b is the housekeeping isoform [33,34]. Finally, SERCA3 isoforms are found in non-muscles such as platelets and epithelial cells [35,36]. The primary structure of SERCA is highly conserved between isoforms, with SERCA2a being approximately 84% similar to SERCA1a and 75% similar to SERCA3 [29]. The differences between isoforms results in different rates of Ca^{2+} transport. For example, SERCA1a has faster Ca^{2+} kinetics, as it possesses a turnover rate approximately two times faster than SERCA2a [37,38]. The maximum velocity of Ca^{2+} transport (V_{max}) is the maximal rate at which SERCA can transport Ca^{2+} into the SR [39]. A higher V_{max} allows for greater Ca^{2+} re-uptake into the sarcoplasmic reticulum, resulting in an improved speed of relaxation [40]. The activity of SERCA2a at submaximal Ca^{2+} concentrations can also be altered through a reduction in Ca^{2+} affinity of SERCA2a [41]. These changes will be discussed in further detail later in this document. Submaximal activity is described by the $Ca_{50,}$ which is defined as the concentration of Ca^{2+} required to achieve 50% of V_{max} (**Figure 1)** [42].

Figure 1. Example SERCA activity curve displaying V_{max} and Ca_{50}.



Cardiac calcium transport is impaired in diabetes

The earliest pathological changes observed in diabetic cardiomyopathy are alterations of Ca^{2+} homeostasis, myocardial structure, and metabolism [21]. These changes lead to an early onset of diastolic dysfunction and eventually systolic dysfunction, even in the absence of ischemia [44]. These negative changes have been observed in both Type I and Type II diabetes [43]. Over time, extended periods of hyperglycemia leads to cellular damage, which can negatively affect the Ca^{2+} transport proteins of the heart resulting in a significantly impaired function [44]. This dysregulation of Ca^{2+} handling, and more specifically, impairment of Ca^{2+} removal from the cytosol, has been observed in both human [45,46] and animal [47] models of heart failure. This impairment of Ca^{2+} handling has been shown to experience a time dependent decay with the progression of the diabetic cardiomyopathy [48]. Diabetic hearts have decreased SERCA2a expression and activity, however, there is currently no data correlating SERCA2a protein levels with glucose

control. In diabetic mice, SERCA2a protein content and maximal activity were decreased by 21% and 32% respectively, leading to diastolic dysfunction [49]. In the diabetic population there is an increased incidence of sudden arrhythmic cardiac death [50]. This increased risk appears to be due to an increased incidence of cellular alternans, which occurs when the heart rate exceeds the ability of the myocardium to cycle calcium on a beat-to-beat basis [51,52]. When SERCA2a was overexpressed by 37% in guinea pig myocytes and intact hearts using an adenoviral vector, cellular alternans was suppressed [51]. This data suggests that SERCA2a may play a key role in sudden arrhythmic cardiac death. Together, these changes suggest that the functional status of SERCA2a may play an important role in the balance between cardiac health and disease.

Increasing cardiac SERCA2a content improves cardiac function

The two main causes of death in heart failure are fatal arrhythmias and declining cardiac output, both of which have been linked with abnormal cardiac calcium homeostasis [53]. Cardiac dysfunction is commonly accompanied by decreased expression and function of SERCA2a [54]. Thus, attempts to restore cardiac function by improving Ca^{2+} handling by increasing SERCA2a function may be a therapeutic approach for heart failure. It is well established that SERCA2a content is strongly associated with cardiac function and left ventricular (LV) contractility. For example, transgenic mice with an overexpression of SERCA2a demonstrated improved Ca^{2+} handling and contractile parameters with improvements in both intracellular calcium decline and myocyte lengthening [55–57]. Further, in heterozygous (SERCA2a+/-) mice where SERCA2a levels were reduced by ~50% the progression to heart failure in response to pressure overload was more rapid than in wild type mice [58]. In humans with dilated cardiomyopathy in

which there is significant dysfunction of the LV, SERCA2a activity has been correlated with contractility [59]. In a study using transgenic, streptozotocin injected type 1 diabetic mice, SERCA2a levels were reduced by 60% compared to control with an accompanying 66% reduction in Ca^{2+} removal. When SERCA2a was conditionally overexpressed in these mice with established diabetic cardiomyopathy using doxycycline based inducible SERCA2a expression, both SERCA2a protein content and LV contractility were normalized [44]. Liraglutide is an oral anti-hyperglycemic agent that has increased in popularity due its beneficial effects on cardiovascular outcomes [60]. This effect may be due to the ability of liraglutide to increase SERCA2a content in cardiomyocytes and, as a result, protect against ischemia/reperfusion injury. More recently, targeting cardiac SERCA2a content as a therapeutic approach to treating heart failure has demonstrated positive results in humans. Specifically, the Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trial utilized an adenoviral vector delivered by intracoronary infusion to over express SERCA2a in the hearts of 39 patients with chronic heart failure. Three concentrations (low dose, $6x10^{11}$ DNase-resistant particles; mid dose, $3x10^{12}$ DNase-resistant particles; high dose, $1x10^{13}$ DNase – resistant particles) of treatment were compared to a placebo group to establish an effective dose and measure outcomes. The CUPID trial demonstrated the safety of this particular gene therapy and resulted in improved left ventricular end systolic and diastolic values as well as functional capacity of patients [61]. Additionally, risk of receiving a left ventricular assist device (LVAD), transplant, or death were reduced by 88% with high dose AAV1/SERCA2a [61]. Long-term follow up after 3 years showed that the number of cardiovascular events and cardiac deaths were lowest in the high-dose intervention group

as compared to control, with a reduction of 82% over a three-year follow-up. No safety concerns arose during the three-year follow-up, as there were no increases in adverse events compared to placebo, further supporting the efficacy of this treatment. In fact, due to the success of the trial, CUPID 2 was conducted to further assess the efficacy of this treatment. CUPID 2 was a phase 2b, double-blind, placebo-controlled, multinational, multicenter, randomized trial consisting of 250 patients with moderate to severe heart failure, defined as New York Heart Association (NYHA) functional class II to IV [62]. Although CUPID 2 did not meet its primary endpoint, it was reported that the proportion of empty viral capsids was significantly lower in CUPID 2 (25%) compared to CUPID (85%) which may have negatively affected transduction efficiency because there were less empty capsids to reduce the activity of neutralizing antibodies on the active capsids [63]. Indeed, a study published while CUPID 2 was being conducted demonstrated that the ratio of full/empty capsids is crucial to the success of the gene transfer [64]. The success of the CUPID trial demonstrated that Ca^{2+} handling and cardiac function can be improved by increasing the quantity of SERCA2a in cardiac tissue. However, the failure of CUPID2 demonstrates that there are limitations to using viral approaches in the heart. Therefore, it is important to investigate alternative strategies. The clinical data supports the evidence observed in animal models indicating SERCA2a is a therapeutic target in models of diabetic cardiomyopathy.

Although it is not fully understood what factors influence SERCA2a protein content in the heart, myocardial SERCA2a content is strongly associated with mitochondrial transcription factor A (TFAM) and 2b (TFB2M) content, with overexpression of these factors resulting in increased SERCA2a content [65]. Diabetes

reduces TFAM levels in the heart, which reduces SERCA2a protein content [66,67]. TFAM content and activity is reduced in diabetic cardiomyopathy and in heart failure heart [65,68]. Additionally, in a rat model of myocardial infarction, both TFAM and SERCA2a mRNA were decreased post-infarct [65]. Exercise training may improve SERCA2a content through the TFAM and TFB2M pathway [65]. Due to the intense metabolic demand of aerobic exercise, exercise stimulates mitochondrial biogenesis, and concordantly, is correlated with increased levels of the mitochondrial transcription factors TFAM and TFB2M. As SERCA2a requires the highest free energy content compared to other ATPases, it is logical that regulation of SERCA2a is closely associated with mitochondrial function and energy production [65]. Exercise training also appears to attenuate the hyperglycemia induced decrease in TFAM protein, as was demonstrated by having male rats perform a 14-week aerobic exercise intervention [69]. This suggests that exercise may exert its effects on SERCA2a protein content by both reducing hyperglycemia and enhancing TFAM and TFB2M protein content.

Adiponectin also appears to regulate SERCA2a content, as was demonstrated by an increase of ~30% in SERCA2a content when H9C2 cardiomyoblasts were treated with adiponectin enriched media. Adiponectin levels are decreased in diabetes and have been shown to have an inverse relationship with the degree of insulin resistance [70,71]. SERCA2a content is also positively regulated by thyroid hormone (T4). When T4 and T3 are low, as in hypothyroidism, SERCA2a content is reduced. Conversely, in hyperthyroidism, SERCA2a levels and cardiac contractility are increased [72]. T3 is able to act on SERCA2a at a transcriptional level by binding to nuclear thyroid hormone receptors and increasing the transcription of the SERCA2a gene [73,74]. It is clear that there

is a relationship between thyroid disorders and diabetes, although the pathophysiology is unknown [75]. Diabetics have a higher prevalence of thyroid dysfunction than the general population, which could lead to reduced SERCA2a [76]. Thus, diabetes negatively affects SERCA2a protein content through reductions in TFAM, TFB2M, adiponectin, and T3. It is possible that other pathways impacted by diabetes may also regulate SERCA2a protein content.

Phospholamban and sarcolipin are regulators of SERCA2a activity

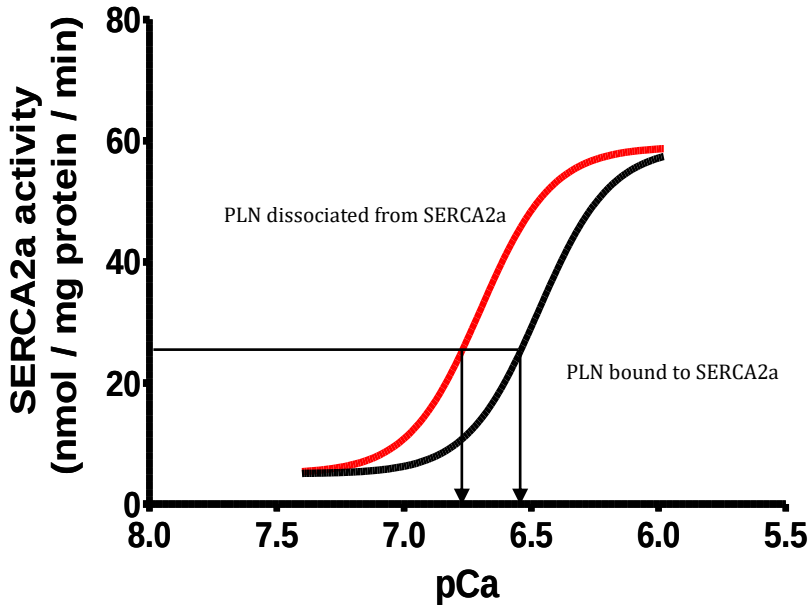
While the total protein content of SERCA2a present in cardiac tissue has a direct effect on overall cardiac function, there are also several regulatory proteins that modify the activity of SERCA2a in cardiac tissue, resulting in altered Ca^{2+} cycling kinetics such as V_{max} or Ca_{50} [77]. Phospholamban (PLN) is a 52 amino acid protein located in the sarcoplasmic reticulum that inhibits SERCA2a at submaximal Ca^{2+} concentrations and is highly expressed in cardiac muscle [78]. The degree to which PLN interacts with SERCA2a is controlled through its phosphorylation status. In the dephosphorylated state, PLN interacts with SERCA2a, resulting in a reduced affinity of SERCA2a for Ca^{2+} (**Figure 2)** [42]. SERCA2a inhibition by PLN occurs when cytosolic Ca^{2+} levels are low, when resting Ca^{2+} concentrations are ~10 nM [42,79]. This inhibitory effect is relieved at high Ca^{2+} concentrations, ~1 μM, when PLN becomes phosphorylated through one of two pathways [79]. At high Ca^{2+} concentrations, Ca^{2+}/calmodulin kinase (CaMKII) phosphorylates PLN at the threonine[17] residue [78]. Alternatively, PLN can become phosphorylated at the serine[16] residue when β-adrenergic signaling increases cyclic AMP (cAMP), leading to the activation of protein kinase A (PKA) [80,81]. In either case,

SERCA2a activity at submaximal Ca^{2+} concentrations is increased, leading to enhanced rates of contraction and relaxation [28,8227,82]. Exercise appears to improve the function of SERCA2a through modulating the relationship between SERCA2a and PLN. Kemi *et al.* [83] demonstrated an exercise induced increase in the SERCA2a/PLN ratio through increased SERCA2a content with no accompanying change in the PLN content. This relationship appeared to be further improved through the CaMK mediated phosphorylation of PLN [83]. Another study demonstrated that exercise training increased the level of phosphorylated PLN, thereby relieving the inhibitory effects on SERCA2a [84]. Consistent with the results seen in other studies SERCA2a expression and Ca^{2+} kinetics were also improved with exercise [84]. Thus, it appears that some of the functional improvement that occurs as a result of exercise is due to a blunting of the inhibitory effect of PLN. Conversely, diabetes can lead to higher levels of SERCA2a inhibition by PLN [85]. Finally, while CaMK and PKA activities appear to be increased in diabetes, lower levels of phosphorylated PLN are observed, which may be due to increased protein phosphatase activity [86]. As a result, PLN can more effectively inhibit SERCA2a activity in diabetic models. Interestingly, the lipid lowering medication, pravastatin, was able to reduce post-MI ventricular tachycardia and Ca^{2+} alternans in part by reducing the amount of active protein phosphatase and restoring Ca^{2+} handling which resulted in higher levels of phosphorylated PLN in pravastatin treated mice [50].

Therapeutic approaches that target PLN have yielded positive results for improving heart function. When recombinant adeno-associated virus (AAV) was used to deliver a pseudophosphorylated mutant of PLN to the hearts of cardiomyopathic hamsters, they experienced increased Ca^{2+} uptake and halted progression of systolic

dysfunction by restoring LV contractility [87]. Pharmacological interventions aiming to improve the function of endogenous SERCA2a proteins can also enhance Ca^{2+} cycling. For example, istaroxime has been shown to restore SERCA2a function in failing hearts and optimize Ca^{2+} reuptake [88]. This effect appears to occur through the ability of istaroxime to effectively dissociate PLN from SERCA2a and thus, relieve the inhibitory effects of PLN. Interestingly, the beneficial effects of istaroxime appear to be due to both an increase in V_{max} [89] and Ca^{2+} affinity [90]. Additionally, istaroxime increases intracellular Na^+ by inhibiting Na^+/K^+ ATPase, leading to a decrease in activity of the Na/Ca^{2+} exchanger and higher cytosolic Ca^{2+}. Together, these produce effects a luso-inotropic action and improve cardiac function [91]. Thus, it is evident that a drug therapy that improves the function of existing SERCA2a is also a potential method to improve Ca^{2+} regulation *in vivo*. In addition to drug therapy, genetic manipulation of PLN can have a beneficial effect on endogenous SERCA2a as was previously discussed.

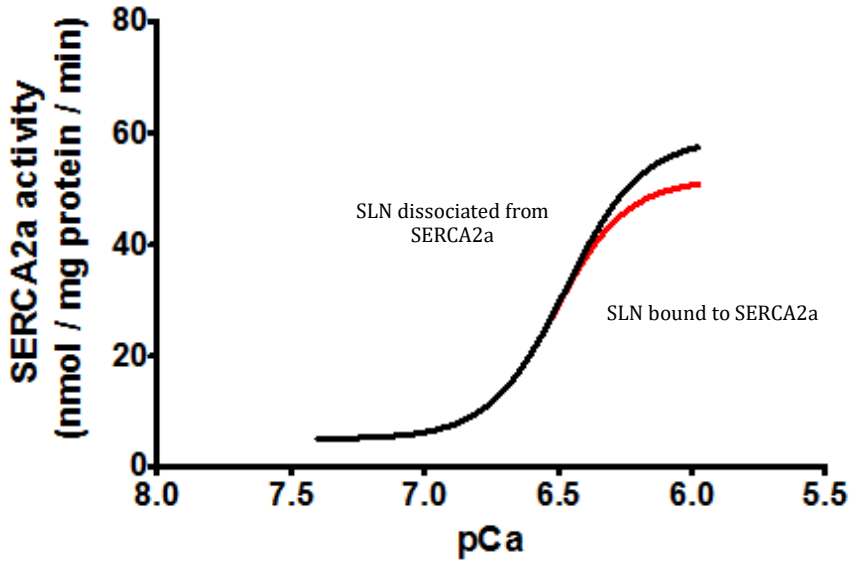
Figure 2. Example SERCA2a activity curve demonstrating the increased Ca_{50} due to the binding of PLN, resulting in a decreased affinity of SERCA2a for Ca^{2+}.



Sarcolipin (SLN) is a 31 amino acid homologue of PLN that regulates SERCA2a function in a similar manner as PLN, by binding directly to SERCA2a [92]. In fact, it appears that both SLN and PLN bind in the same molecular groove within the SERCA2a protein structure [78]. However, the mechanism through which SERCA2a function is altered appears to be slightly different. While PLN primarily alters affinity for Ca^{2+}, SLN decreases SERCA2a activity by decreasing ATP binding capacity of SERCA2a [93], resulting in a reduced V_{max} [78] (**Figure 3**). A mouse model suggested that the presence of these regulatory proteins appears to be chamber specific, with PLN being the predominant regulator in the ventricles while SLN is primary regulatory protein in the atria [82]. In humans, the highest expression of SLN is found in skeletal muscle, while rodents have higher expression of SLN in cardiac muscle [78,94]. Despite the attenuated levels of SLN protein expressed in human heart tissue compared to skeletal muscle, SLN still appears to play an important role in the balance between health and disease [95]. It was recently demonstrated that SLN becomes dysregulated in humans with cardiovascular disease [96]. Left ventricular tissue removed from patients undergoing surgery for mitral valve regurgitation showed that SLN mRNA and protein level were increased 12- and 6-fold, respectively compared to healthy controls [96]. In a mouse SLN knockout model, KO mice had higher SERCA2a activity and contractility in the atria but not the ventricles, compared to WT [97]. Conversely, when SLN was overexpressed in mouse ventricles, Ca^{2+} transport and rate of relaxation decreased accordingly [98]. While it is known that SLN can modulate SERCA2a activity, its role in heart failure is less clear than that of PLN. This is because there is a differential expression in small mammals, where SLN is found primarily in the atria with low levels in skeletal muscle and ventricles, and large

mammals, where the largest quantity is in fast twitch muscle rather than atria or ventricle [98]. As the role of SLN in cardiac contractility appears to be primarily in the atria and not the ventricles, there have been no attempts to therapeutically manipulate it.

Figure 3. Example SERCA2a activity curve demonstrating the resultant decrease in maximal activity due to SLN binding.



Post-translational modifications regulate SERCA2a activity

Post-translational modifications change the activity of proteins. SERCA2a activity is regulated by several post-translational modifications including Glutathionylation [99], Nitration [100], Glycosylation [101], *O*-GlnNAcylation [102], SUMOlyation [103,104], and Acetylation [103,105]. These post-translational modifications are outlined in **Table 1**. It was previously thought that SERCA2a might be regulated by phosphorylation like other P-type ATPases, such as the Na^+/K^+ [106]. Naraynan and Xu described a phosphorylation of SERCA2a at serine 38 with an increase in V_{max} [107]; however, that result has not been replicated by other investigators [108]. Further studies using isolated cardiomyocytes,

enriched sarcoplasmic reticulum vesicles and purified SERCA2a protein demonstrated that the reported phosphorylation of SERCA2a is likely to be an unrelated phosphoprotein and that serine 38 of SERCA2a is not a target for phosphorylation [109,110].

Glutathionylation occurs when a disulfide bond is formed between a cysteine residue of a protein and a glutathione molecule. SERCA2a can be glutathionylated at the cysteine^{674} residue, and when this occurs, SERCA2a activity is increased by approximately 40% [111,112]. In atherosclerotic conditions, cysteine^{674} becomes permanently oxidized which prevents glutathionylation and reduces the activation of SERCA2a [112].

Nitration involves the addition of a nitro group to SERCA2a. Nitration of SERCA2a leads to a drop in activity of approximately 25% in both slow twitch skeletal muscle and high glucose perfused rat hearts [113,114]. SERCA2a nitration has been demonstrated in the hearts of humans with dilated cardiomyopathy, in which the level of SERCA2a nitration was shown to be double that of age-matched controls [115]. Additionally, in rat hearts that were perfused with high glucose, the level of SERCA2a nitration nearly doubled [114]. While nitration of SERCA2a has been reported, the role that it plays on the regulation of SERCA2a activity has not been determined.

Glycosylation occurs when a saccharide is attached to a protein. Increases in glycosylation have been shown to reduce SERCA2a mRNA and protein by 25-45% while increasing PLN by 40% [116,117]. Together, these effects reduce the Ca^{2+} pumping of SERCA2a, leading to a 35% decrease in relaxation rates which were used as an indirect measure of SERCA2a [117]. This modification occurs when glucose levels are high,

therefore it is of interest when examining the relationship between diabetes and heart disease [116,117].

O-GlnNAcylation is a form of glycosylation in which a single O-linked *N*-acetylglucosamine is attached to a serine of threonine residue [116,118]. *O*-GlnNAcylation of SERCA2a reduces SERCA2a activity by modifying SERCA2a directly as well as modification of PLN, resulting is less phosphorylated PLN [116,117,119]. Rat cardiomyocytes exposed to high glucose experienced a substantial increase in level of nuclear *O*-GlnNAcylation with an accompanying decrease of 28-37% in SERCA2a mRNA and 25% reduction in SERCA2a expression although they did not measure corresponding changes in SERCA2a activity [116].

Table 1. Known posttranslational modifications of SERCA2a.

Post translational modification	Regulatory Process	Effect on SERCA2a
Glutathionylation	Disulphide bond at cysteine[674] residue[120]	Increased V_{max} of SERCA2 by ~40%[121]
Nitration	Addition of Nitro group to SERCA2a[100,113]	Decreased V_{max} of SERCA2a by ~25%[100,113]
Glycosylation	Addition of carbohydrate group to SERCA2a[101]	Decrease in SERCA2a mRNA and total protein expression of 37% and 30% respectively[117,122]
O-glnNAcylation	N-acetylglucosamine added to serine or threonine residue[123]	Reduced SERCA2a protein expression by 25%[122]
SUMOlyation	Addition of SUMO1 to lysine[480] and lysine [585] residues[103]	Increases V_{max} of SERCA2a by improving ATP binding affinity[103,104]
Acetylation	Addition of an acetyl group to SERCA2a, potentially at lysine[464], lysine[510], and lysine[533] residues[105]	Likely decreases V_{max} of SERCA2a by decreasing ATP binding affinity[103,105]

SUMOylation of SERCA2a occurs when the small ubiquitin-like modifier-1 (SUMO1) is ligated to the lysine^{480} and lysine^{585} residues of SERCA2a [103]. SUMO1 has been suggested to have cardioprotective properties in the heart. A study by Kho et al. showed that in mice with heart failure, total SUMO1 levels decreased by 30-40% with a corresponding, although unquantified by the authors, decrease in levels of SUMOylated SERCA2a [103]. To further support this, when SUMO1 expression was increased using lentiviral gene transfer in the pressure-overloaded heart of mice, both cardiac performance and SERCA2a function were rescued. This increase in SUMO1 led to increased half-life from 4.9 to 5.9 days of SERCA2a as well as increased ATP binding affinity, demonstrating a role of SUMO1 on protein stability and function [103]. Additionally, SUMO1-AAV gene transfer in a pig model of ischemic heart failure successfully improved cardiac function [104]. Mechanistically, SUMO1 appears to improve SERCA2a function by increasing its ATP binding affinity [103]. Additionally, it has been suggested that the beneficial effects of SUMO1 are due to the fact that SUMO1 blocks other post-translational modifications that occur at lysine residues, such as acetylation [124]. More recently, the small molecule N106 was shown to increase SUMOylation of SERCA2a resulting in increased SERCA2a activity in a dose-dependent manner [125]. The maximal dose resulted in a 200% increase in both SUMOylation of SERCA2a and SERCA2a activity. This increase was accompanied by an increase in the contractile properties of rat cardiomyocytes and improved ventricular function in mice subjected to trans aortic constriction [125,126].

Acetylation

Histone acetylation is a modification of chromatin structure that is involved in controlling DNA transcription, replication, and repair [127]. Acetylation takes place at lysine residues where the addition of an acetyl group prevents the formation of a positive charge on the amino group (**Figure 4**). Generally, acetylation of histones by histone acetyltransferases (HATs) leads to a decondensed chromatin and a more transcriptionally active state [128]. Conversely, when an acetyl group is removed from a histone by histone deacetylases (HDACs), the DNA becomes wound more tightly, ultimately leading to reduced transcription of that particular region [129]. In the long term this alteration of transcription results in altered protein production. It is now known, that despite their name, HATs and HDACs also act on non-histone targets and that lysine acetylation is involved many cytoplasmic processes and many nuclear functions [130]. Acetylation of non-histone targets was first observed by p300/CBP, which is capable of acetylating the tumor suppressor transcription factor p53 at multiple sites [131]. This acetylation process results in increased DNA binding activity by p53 and a resulting increase in the activation of its gene targets [128]. The discovery that acetylation can effectively alter protein activity resulted in a drastic change in the traditional view of protein regulation. However, whether acetylation of a protein activates or inhibits its activity is protein dependent. For example, when PGC-1α is acetylated by GCN5 it is deactivated [132,133]. Acetylation of proteins appears to be linked to cellular energy status, where a high level of carbon flux leads to acetylation of proteins and the promotion of an anabolic state and energy storage [134].

To gain a greater understanding of non-histone protein acetylation in cell signaling, broad scale proteomic approaches have characterized acetylation sites in human cells using mass spectrometry. Choudhary *et al.* [130] identified 3600 acetylation sites in 1750 different proteins in human MV4-11 cells, which are derived from macrophages. Foster *et al.* used a similar approach in guinea pig heart tissue to probe for acetylated proteins. A total of 994 acetylation sites on 240 proteins were identified [105]. Interestingly, 59% of acetylated proteins were mitochondrial, suggesting that acetylation may have a key role in the regulation of metabolism [105]. Another significant finding of the Forster *et al.* study was that SERCA2a is acetylated at three sites (lysine^{464}, lysine^{510}, and lysine^{533}). Thus, it is possible that SERCA2a activity may be regulated by its acetylation status [105]. However, this hypothesis has yet to be tested. Kho *et al.*[103] reported that acetylated SERCA2a was more prominent in failing hearts, which suggests that the acetylation of SERCA2a may play a role in heart failure. However, they did not present data to support this observation. Thus, the regulation of SERCA2a activity by acetylation has yet to be directly examined. We have generated preliminary data suggesting that the activity of constitutively acetylated SERCA2a is 30% lower than the wild type SERCA2a (**Figure 5**). As a result, pharmacological manipulation of SERCA2a acetylation may be an effective method to improve SERCA2a function. However, there is currently very little definitive information regarding how SERCA2a is post-translationally modified in any model, including models of diabetes. We have preliminary data showing that acetylation of SERCA2a is increased twofold in streptozotocin injected Sprague-Dawley rats, which is a model of type 1 diabetes characterized by impaired

SERCA2a activity and left ventricular dysfunction (**Figure 6**). However, the HATs and HDACs that regulate the acetylation of SERCA2a are still unknown.

Figure 4. Illustration of protein acetylation/deacetylation of target proteins.
Histone deacetylases act to remove acetyl groups from target proteins. Conversely, histone acetyltransferase adds acetyl groups to target proteins. The balance of deacetylase and acetyltransferase activity determines the acetylation state of a protein.

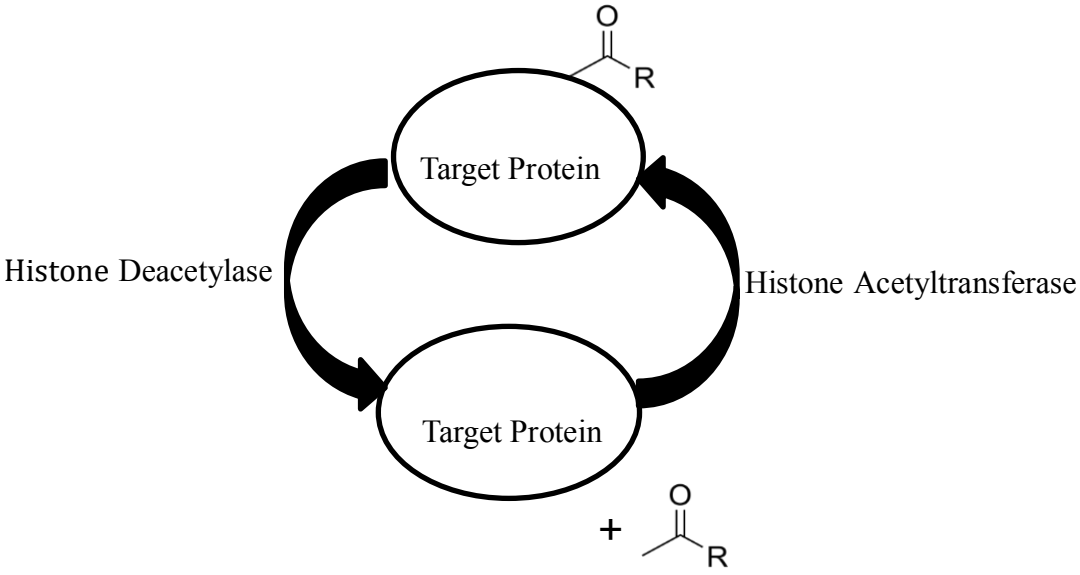


Figure 5. Activity of constitutively acetylated SERCA2a samples from two trials (n=5) were harvested 48 hours after transfection.
Maximal activity of the constitutively acetylated SERCA2aK464Q/K510Q/K533Q mutant was 35% lower than that measured for the wild-type SERCA2a; whereas, the activity of the acetylation deficient SERCA2aK464R/K510R/K533R mutant did not differ from the wild-type SERCA2a. *, different from Control ($P<0.05$). #, different from Acetylation-mimic SERCA2a$^{K464/510/533Q}$ mutant ($P<0.05$). Note: This figure has been adapted with permission from Susser [135].

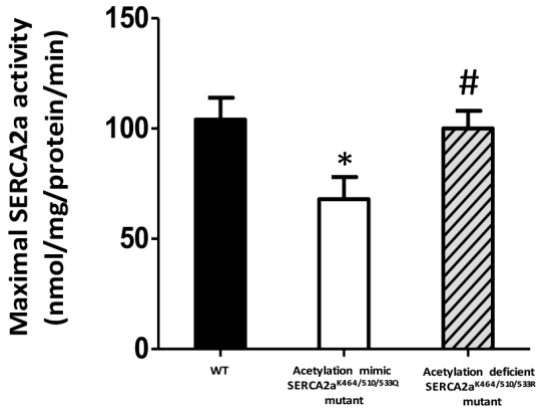
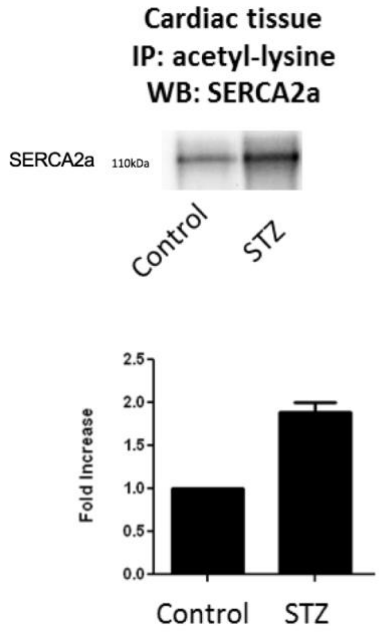


Figure 6. Preliminary data indicating that SERCA2a acetylation is increased by 2-fold in the hearts of streptozotocin injected Sprague-Dawley Rats ($p<0.05$).
This is a model of type 1 diabetes characterized by impaired SERCA2a activity and left ventricular dysfunction. Immunoprecipitation was performed using acetyl-lysine antibody followed by western blotting using an anti-SERCA2a antibody. Note: This figure has been adapted with permission from Susser [135].



Histone deacetylases

There are currently 18 known HDACs, which can be divided into two distinct groups, as outlined in **Table 2.**[127] The first group is a family of zinc-dependent HDACs that are structurally related to the yeast Hda1/Rpd3 proteins, and are considered to be the "classical" HDACs. The second group of HDACs, the NAD^+-dependent sirtuin family, are mammalian homologues of the Silent information regulator 2 (Sir2) protein and were originally discovered in yeast [136,137]. The sirtuins will be discussed in further detail in the following section. These two groups can be further divided into four classes based on the level of sequence conservation compared to their yeast counterparts. Class I consists of HDAC1, HDAC2, HDAC3, and HDAC8, which are most closely related to yeast RPD3

and are localized primarily within the nucleus [138]. Class II is made up of two subclasses: subclass IIa, which contains HDAC4, HDAC5, HDAC7, and HDAC9 and subclass IIb containing HDAC6 and HDAC10 [136]. Class II HDACS are related to yeast HDA1 and are able to shuttle between nucleus and cytoplasm [138]. Class III is made up of sirtuins 1-7, while the only member of Class IV is HDAC11[127].

Table 2. HDACs by class, cellular location, and compound(s), which alter activity.

HDAC Class	**HDACs**[138]	**Cellular Location**[138,139]	**Inhibitor**[140–142]	**Activator**[143]
Class I	HDAC1, HDAC2, HDAC3, HDAC8	Nucleus	Trichostatin A, FK228	
Class IIA	HDAC5, HDAC7, HDAC9	Nucleus and cytoplasm	Trichostatin A, MC1568	
Class IIB	HDAC6, HDAC10	Cytoplasm	Trichostatin A, Bufexamac	
Class IV	HDAC11	Nucleus and cytoplasm		
Sirtuins	SIRT1-7	Nucleus, cytoplasm, and mitochondria	Nicotinamide	NAD^+

Inhibitors of HDACs were discovered before the HDACs themselves were discovered [136]. HDACs inhibitors can be divided by their structural classes which include

hydroxamates, cyclic peptides, aliphatic acids, and benzamides [138]. While these inhibitors are effective on the classical HDACs, they are ineffective in inhibiting the sirtuin deacetylases, which do not contain a zinc-binding site. In contrast, the Sirtuins can be inhibited broadly by nicotinamide, a metabolite of NAD^+ [140]. These inhibitors can cause cell cycle arrest, apoptosis, senescence, production of ROS and mitotic cell death [144]. A variety of HDAC inhibitors have shown promise as anti-cancer agents [144]. More recently, HDAC inhibitors have also shown promise in the treatment of infectious diseases [145] and prevention of pathological remodeling of the heart [146,147]. Due to these promising clinical effects, novel inhibitors are continuing to be developed and characterized. The HDACs targeted by commonly used inhibitors are listed in **Table 2.**

Sirtuins have a role in the balance between health and disease

Of all the deacetylases, the sirtuin family of deacetylases are some of the most interesting due to their apparent beneficial role in aging and metabolism [139]. The sirtuin family is outlined in **Table 3.** Sirtuins can be activated by physiological interventions such as caloric restriction and exercise or by pharmacological methods and have been shown to extend the lifespan and improve health in a variety of organisms from yeast to mammals [148]. The beneficial effects of the NAD^+ precursor nicotinamide riboside on mouse muscle stem cells were attenuated when SIRT1 was knocked out [149]. Given this promising data, the beneficial therapeutic effects of the NAD^+-sirtuin axis cannot be ignored [150].

Table 3. Location of sirtuins within the cell.

Sirtuin	Location[148,151,152]
SIRT1	Nucleus, Cytoplasm
SIRT2	Cytosol
SIRT3	Mitochondria, Cytosol, Nucleus
SIRT4	Mitochondria
SIRT5	Mitochondria
SIRT6	Nucleus
SIRT7	Nucleus

Sirtuins also appear to be relevant in the pathogenesis of heart disease, with the effects of SIRT1 and SIRT3 being the most extensively studied forms in the cardiovascular system [151]. Additionally, SIRT6 has been shown to prevent the development of cardiac hypertrophy [153]. In the cardiomyocytes derived from calorically restricted rats, expression of SIRT1-4 and -7 is increased while the expression of SIRT5-6 is unchanged [154]. SIRT1 is primarily nuclear, but can shuttle between nucleus and cytoplasm [155]. SIRT2 is located exclusively in the cytoplasm [154]. SIRT3 has been detected in the nucleus, mitochondria, and cytoplasm [152]. SIRT4-5 are strictly found in mitochondria [148] while SIRT6-7 have only been detected within the nucleus [148], therefore, it is unlikely that SIRT4-7 are responsible for the deacetylation of SERCA2a based on their subcellular distribution.

In mice subjected to transverse aortic constriction, SIRT1 overexpression attenuated fibrosis, cardiac hypertrophy, and dysfunction as compared to the non-

transgenic control. However, high levels of SIRT1 overexpression stimulated cardiomyopathy [156]. It is possible these deleterious effects occurred due to excessive NAD^+ consumption by extreme levels of SIRT1 leading to ATP deficiency. SIRT1 heterozygous mice develop obesity and insulin resistance while SIRT1 overexpression leads to decreased fasting serum insulin, glucose, and cholesterol [157,158]. SIRT1 has also been shown to increase in response to exercise in the heart and skeletal muscle of rats [159,160]. Further, SIRT1 plays a role in improving cardiac function in a diabetic cardiomyopathy by increasing SERCA2a expression [161]. The beneficial effects of metformin, a commonly prescribed diabetes drug, appear to be through activation of the SIRT1 pathway [162].

The activity of another member of the sirtuin family, SIRT3, also appears to have a positive effect on heart structure and function. Phenotypically, SIRT3 KO mice are similar to SIRT1 deficient mice but experience an increase in obesity, insulin resistance, and hyperlipidemia in response to a high fat diet [163]. When SIRT3 null mice were exposed to stress stimuli they experienced significant negative cardiac hypertrophic effects, while mice expressing SIRT3 were protected [164]. Additionally, the hearts of SIRT3 knockout mice experienced significantly less recovery of cardiac function after exposure to ischemia than their wild-type (WT) counterparts [165]. High fat feeding results in the hyperacetylation of mitochondrial proteins and a downregulation of SIRT3 in wild-type mice [166]. SIRT3 have been shown to play a role in human health and increased in the skeletal muscle of overweight adolescents in response to exercise, even without caloric restriction [167]. Sirtuin levels have been shown to be decreased by diabetes, aging and obesity [168].

Histone acetyltransferases

HATs use acetyl-coenzyme A (acetyl-CoA) as a cofactor to add an acetyl group to a lysine residue. To date, more than 22 different HATs have been discovered in human and murine cells, which have been divided into 3 categories based on structural characteristics: GNAT, p300/CBP, and MYST [169]. The better characterized HATs are listed in **Table 4.**

Table 4. Histone acetyltransferases and their classifications.

HAT Family	**HATs**[134,169,170]
GNAT	GCN5, GCN5L1, PCAF, Hat1, Elp3 Hpa2
MYST	Tip60, MOZ, Sas 2, Sas3, MOF, Esa1, MORF, Hbo1
p300/CBP	p300, CBP, Rtt109

One histone acetyltransferase of particular interest is general control non-depressible 5 (GCN5). Increased interaction of GCN5 with PGC-1α occurs during caloric excess leading to increased PGC-1α acetylation and a reduction in its transcriptional activity. In contrast, caloric restriction results in reduced GCN5 interaction with PGC-1α resulting in its decreased acetylation and increased activity [171]. PGC-1α has been shown to be regulated through phosphorylation and acetylation [172]. The actions of GCN5 have been shown to be opposed by SIRT1 [173–175]. PGC-1α can be deacetylated by SIRT1 and SIRT3, leading to its activation [172,176]. PGC-1α can also be phosphorylated by AMP-activated protein kinase (AMPK), which stabilizes the protein [172]. AMPK can then be activated by SIRT1 and SIRT3 and once AMPK is activated, it

can phosphorylate liver kinase B1 (LKB1) [177,178]. LKB1 then phosphorylates and activates AMPK [177,178]. AMPK can also activate SIRT1 through its role in elevating NAD^+ levels, suggesting that these proteins have a synergistic relationship [179]. Exercise also appears to increase in PGC-1α activity as a result of decreased interaction with GCN5 [180]. It is currently unknown if GCN5 interacts with SERCA2a.

The HAT p300 has been shown to readily undergo autoacetylation when high levels of acetyl-CoA are present [181]. Further to this, high glucose conditions have been shown to increase the activity of p300, which can then acetylate SIRT2 and decrease its activity [182,183]. This relationship appears to be reciprocal, with SIRT2 deacetylating p300 resulting in decreased p300 activity [181]. Because of this, p300 is another HAT of particular interest in diabetes, where systemic glucose levels are dysregulated. Additionally, p300 has been shown to acetylate PGC-1α [184]. Another HAT that readily undergoes autoacetylation resulting in increased activity is Tip60 [185]. Deacetylation of Tip60, and thus, lower acetylation of its downstream targets has been shown to occur through the actions of SIRT1 [185,186]. In addition to regulating the activity of Tip60, SIRT1 has also been shown to deacetylate proteins that have been acetylated by Tip60, such as histone H4K16 and lysine[120] of p53 [187,188].

Very little information is currently available regarding HAT inhibitors, particularly on their site specificities. Anacardic acid has been shown to be a non-specific, broad spectrum HAT inhibitor [136,189]. Various derivatives of anacardic acid have been synthesized in order to find HAT inhibitors with more specific actions [190]. One such derivative is MG149, which has been shown to selectively inhibit Tip60 and MOZ, which are members of the MYST family [191]. p300/CBP inhibition has also been achieved

through the use of a small molecule inhibitor, C646 [192]. Finally, the GCN5 network has been shown to be effectively inhibited by CPTH2 [193,194]. However, it is currently unknown if HAT inhibitors influence SERCA2a acetylation.

Chapter 2: Study Design and Methods

Statement of problem

While it is currently known that SERCA2a can be acetylated [105], it is still unclear which HDAC(s) and HAT(s) are responsible for this posttranslational modification of SERCA2a. We know that high glucose increases acetyltransferase activity and reduces deacetylase activity, but we do not know if high glucose results in increased acetylation of SERCA2a. As such, we intend to address these literature gaps by examining three objectives:

1) To determine if high glucose exposure alters the acetylation level of SERCA2a;
2) To identify the HDAC(s) responsible for the regulation of SERCA2a acetylation; and,
3) To identify the HAT(s) responsible for the regulation of SERCA2a acetylation under high glucose conditions.

Our strategy for objective 1 was to isolate primary rat cardiomyocytes and incubate them with high glucose (25 mM) media to examine potential changes in SERCA2a acetylation. To accomplish objectives 2 and 3, we used small molecule inhibitors of HDACs and HATs in a rat primary cardiomyocyte model to examine the pathways involved in SERCA2a acetylation.

Hypotheses

We hypothesized that increased SERCA2a acetylation will have a negative effect on SERCA2a function and that acetylation of SERCA2a is regulated by the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs.)

Three specific hypotheses were tested:

1) High glucose exposure increases the acetylation of SERCA2a
2) The HDAC that deacetylates SERCA2a is of the sirtuin family.
3) That the HAT GCN5 acetylates SERCA2a after high glucose exposure.

Objective 1: Our general approach to determine if high glucose alters SERCA2a acetylation was to culture primary rat cardiomyocytes in the presence of high glucose (25 mM) media. We measured the changes in acetylation after exposing the cardiomyocytes to glucose at 1, 2, 4, and 18 hours by Western blotting with an anti-acetylated lysine antibody and measured the levels of the 110 kDa band where SERCA2a resides. We hypothesized that acetylation would progressively increase with the duration of exposure to high glucose. To confirm that the increase in acetylation at the 110 kDa band was indeed resulting from an increase in acetylated SERCA2a, immunoprecipitation was performed after 18 hours of high glucose exposure to pull down the target protein and those samples were then examined by Western blotting.

Cardiomyocyte isolation

Twelve-week-old, adult male Sprague Dawley rats (~ 250 grams) were purchased from Central Animal Care at University of Manitoba. Animals were treated in accordance with the guidelines of the University of Manitoba Animal Protocol Management and Review Committee and the Canadian Council on Animal Care. All rats were fed *ad libitum* with a standard rat chow diet.

Rats were anesthetized by an intraperitoneal injection of ketamine-xylazine (90:100 $mg \cdot kg^{-1}$) and the heart was removed and promptly placed in ice cold phosphate buffered saline (PBS). Extraneous tissue was removed from the heart, which was then secured by the aorta onto the cannula of a Langendorff system using surgical suture. Langendorff perfusion was used as this is the most reliable method to isolate high quality cardiomyocytes [195]. The heart was perfused with oxygenated Ca^{2+} free buffer containing 90 $mmol \cdot L^{-1}$ NaCl, 10 $mmol \cdot L^{-1}$ KCl, 1.2 $mmol \cdot L^{-1}$ KH_2PO_4, 5 $mmol \cdot L^{-1}$ $MgSO_4 \bullet 7H_2O$, 15 $mmol \cdot L^{-1}$ $NaHCO_3$, 30 $mmol \cdot L^{-1}$ Taurine, and 20 $mmol \cdot L^{-1}$ Glucose. After washing, the heart was perfused with 20 mL of Ca^{2+} free buffer supplemented with 100 mg BSA (Sigma-A7030), 32 mg Collagenase Type 2 (Worthington LS004177), and 15 µL of 100mM $CaCl_2 \bullet 2H_2O$ for 30 minutes.

After digestion, atria were removed and discarded. Ventricles were transferred to a 50 mL centrifuge tube where the cardiomyocytes were washed twice with of Ca^{2+} free buffer supplemented with 100 mg BSA (0.2%) and 130 µL of 100 mM $CaCl_2 \bullet 2H_2O$ (260 µM). Cells were then washed once with Ca^{2+} buffer containing 0.4% BSA, 520 µM $CaCl_2 \bullet 2H_2O$, and 2500 U penicillin-streptomycin. Final wash buffer contains 0.4% BSA, 1.04 µM $CaCl_2 \bullet 2H_2O$, and 2500 U penicillin-streptomycin. After the final wash the

buffer was removed and Medium 199 media (Gibco) was added. Cells were then plated on 100 mm Nunclon delta treated cell culture dishes (Thermo Scientific). Media was changed after 2 hours.

High glucose exposure

All cells were cultured in M199 media, which has a glucose concentration of 5 mM. To generate the high glucose condition, additional glucose was added to the M199 media to bring the glucose concentration to 25 mM. This model has previously been used to mimic the elevated glucose levels that are present in severely diabetic rats that results in impaired calcium cycling as occurs with diabetic cardiomyopathy [17,18,116]. To examine the time course of high glucose effects, the glucose concentration was brought to 25 mM for 1 hour, 2 hours, 4 hours, and 18 hours prior to harvest.

Cell collection

Treated cardiomyocytes were collected in PBS and centrifuged for 1 minute at 100 g to pellet. The PBS was then removed. One-hundred and fifty μL of non-denaturing lysis buffer containing 20 mM Tris HCl (pH 8), 137 mM NaCl, 10% Glycerol, and 1% Triton X-100 supplemented with 5 mM nicotinamide and 5 μM trichostatin (deacetylase inhibitors) and protease inhibitors was added, and the pellet was resuspended by vortex. Cells were then incubated on ice for 10 minutes and were spun at 12500 g for 3 minutes to pellet cell debris. The supernatant was transferred to a separate tube and the pellet was discarded. Excess supernatant was snap frozen in an Eppendorf tube using liquid nitrogen.

Western blotting

Total protein content of the lysates was quantified using a DC (detergent compatible) protein assay (Bio-Rad Laboratories). Thirty micrograms of protein were loaded and resolved on 7.5% - 15% SDS-polyacrylamide gels, based on protein size, and transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad Laboratories). Membranes were blocked in 5% BSA in tris-buffered saline tween (TBST) for one hour. After blocking, blots were immuno-labeled with protein specific primary antibodies (e.g. Acetylated Lysine) followed by three 10 minute washes with TBST. Blots were then incubated in specific secondary antibody and washed an additional 3 times in TBST for 10 minutes each. Electrochemiluminescence (ECL) reagents (Bio-Rad Laboratories) were added to membranes and visualized with the Fluor-S-Max MultiImager (Bio-Rad Laboratories). Results of condition were normalized to β-actin followed by normalization to control.

Immunoprecipitation

To determine acetylation status, cardiomyocyte lysates were first immunoprecipated using an anti-SERCA2a antibody (Santa Cruz 8094). Protein G Magnetic beads (Bio-Rad Laboratories) were first washed three times in PBS and 0.1% Tween 20. After washing, beads were coated with 5 µL of primary antibody and rotated end over end for 10 minutes at room temperature. After pre-coating, beads were washed three times in PBS and 0.1% Tween 20. Beads were then added to a volume of lysate containing 500 µg of protein and rotated end over end for 1 hour. Washes were

performed three times in PBS and 0.1% Tween 20. Beads were then incubated at 70°C in 40 µL of 2X Laemmeli buffer (Bio-Rad Laboratories) for 10 minutes to elute the protein bound to the beads. A Western blot with an acetylated lysine antibody (Cell Signalling #9441) was then performed with these samples. Results of each condition were normalized to SERCA2a followed by normalization to control. Samples from multiple conditions were run on a single blot. For this document, two example blots were generated which were then trimmed to display specific conditions in the results section.

Objective 2: The influence of HDACs on SERCA2a acetylation were examined by adding HDAC inhibitors to the rat primary cardiomyocytes for 18 hours in normal glucose (5 mM) media (**Figure 7**). This timeline was chosen based on the results observed in objective 1, which showed that maximal acetylation of SERCA2a occurred at 4 hours and was maintained through to 18 hours when exposed to high glucose. To gain insight as to which HDACs might interact with SERCA2a, 10 mM nicotinamide was used to inhibit the sirtuin class of HDACs. In a second experiment, 1µM Trichostatin A (TSA) was used to inhibit all non-sirtuin HDACs. If TSA caused a change in SERCA2a acetylation, we planned to use 1µM FK 228, 1µM MC 1568 and 500 µM bufexamac to inhibit Class I, IIa, and IIb histone deacetylases, to determine the role of each HDAC class in the increased SERCA2a acetylation (**Figure 8**). However, as TSA did not alter SERCA2a acetylation, these experimental conditions were not performed. Control conditions consisted of normal (5 mM) glucose media without inhibitors. The methods used to obtain cardiomyocytes and measure SERCA2a acetylation in this objective were the same as those described in objective 1. In addition, we also used a SERCA2a activity

assay to determine if SERCA2a activity was altered by any of the experimental conditions.

Measurement of SERCA2a activity

Measurement of SERCA2a activity was performed using the spectrophotometric assay as described by Duhamel *et al.* [77] using a plate reader (SPECTRAmax; Molecular Devices). This assay measures the amount of ATP hydrolysis that occurs when stimulated by different concentrations of Ca^{2+}. The assay buffer was prepared using 4.25 mL ATPase buffer, 15 µL lactate dehydrogenase, 15 µL pyruvate kinase and 8.8 µL ionophore. Ionophore is added to prevent back inhibition by accumulation of Ca^{2+} in the SR [196]. The SERCA2a inhibitor cyclopiazonic acid (CPA) [197] was used in one sample in order to determine background ATPase activity. This value was then subtracted from total Ca^{2+} stimulated ATPase activity, thus allowing the calculation of SERCA2a activity alone. The output values are then used to calculate the kinetic properties of Ca^{2+}-dependent SERCA2a activity. The kinetic parameters of interest are maximal SERCA2a activity, Hill coefficient, and Ca_{50}. This protocol allows for comparison and analysis of 3 samples per plate.

Figure 7. Experimental flow using HDAC inhibitors.
Two hours after plating, media was changed and HDAC inhibitors were added to the cardiomyocytes for 18 hours. Cardiomyocytes were then collected and added to non-denaturing lysis buffer supplemented with deacetylase and protease inhibitors.

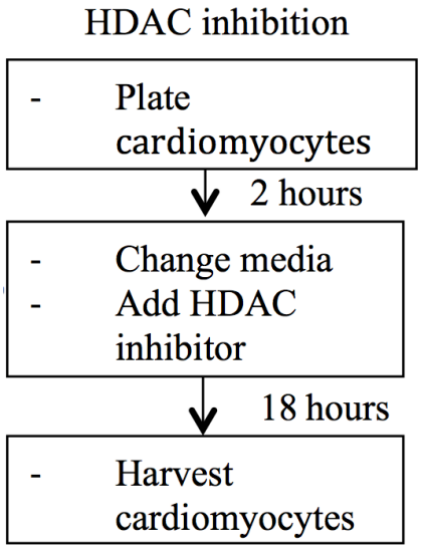
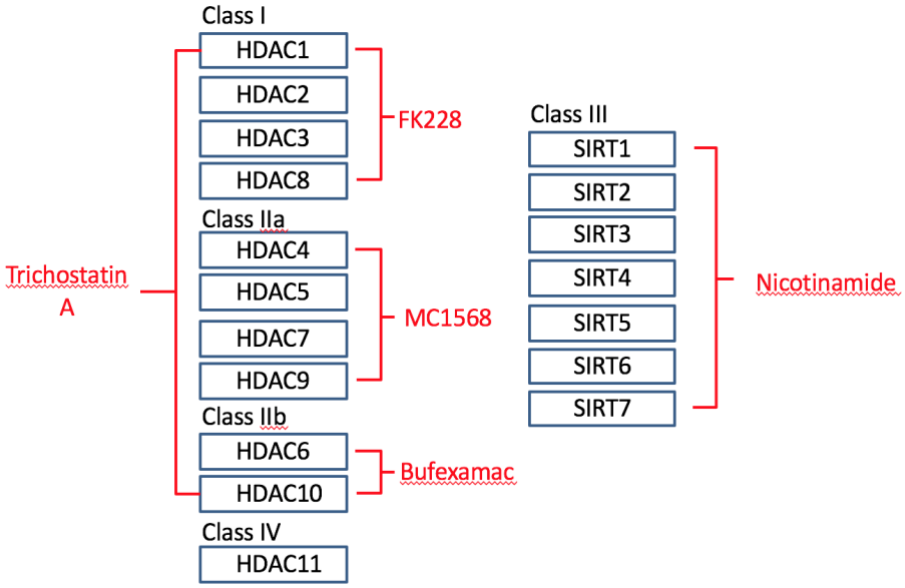


Figure 8. Inhibitors of HDACs.
Trichostatin A was used to inhibit Class I and Class II HDACs that contain a zinc-binding site. If an increase in acetylation was observed, further probing using FK228, MC1568, and Bufexamac would have been used to examine if the HDAC responsible for SERCA2a deacetylation is of Class I, Class IIa, of Class IIb, respectively. Nicotinamide was used to inhibit all sirtuins while NAD^+ was used to activate the sirtuins to evaluate their role in the acetylation status of SERCA2a. There are currently no known chemical activators or inhibitors for Class IV HDACs.



Objective 3: To determine which HAT might interact with SERCA2a, 3 μM anacardic acid was used to broadly inhibit all HATs. Preliminary trials using anacardic acid to broadly inhibit HATs did not result in any differences in SERCA2a acetylation as compared to control. We speculate that the reason for this is likely that, in its baseline state, SERCA2a is minimally acetylated. Thus, we introduced a high glucose stimulus, as we have previously observed that 25 mM glucose results in higher levels of acetylation of SERCA2a. Cardiomyocytes were pretreated for 1 hour with HAT inhibitors prior to the addition of 25 mM glucose. The cardiomyocytes were then exposed to the high glucose conditions in addition to the HAT inhibitor for 18 hours before being harvested. This allowed us to determine which HATs play a role in the acetylation of SERCA2a by looking at which inhibitors prevented the increases induced by high glucose exposure (**Figure 9**). We targeted specific acetyltransferases using 5 μM C646, 1 μM MG149 and 10 μM CPTH2 to selectively inhibit p300/CBP, Tip60 and MOZ, and GCN5, respectively (**Figure 10**). The methods used to obtain cardiomyocytes and to examine SERCA2a acetylation and activity in this objective were the same as previously described in objectives one and two.

Figure 9. Experimental flow using HAT inhibitors.
Two hours after plating, media was changed, and HAT inhibitors were added to the cardiomyocytes for 1 hour. Following pre-treatment, glucose was added to bring glucose concentration to 25 mM for 18 hours. Cardiomyocytes were then collected and added to non-denaturing lysis buffer supplemented with deacetylase and protease inhibitors

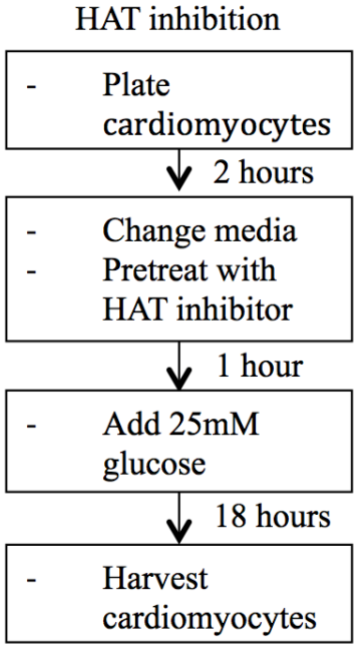
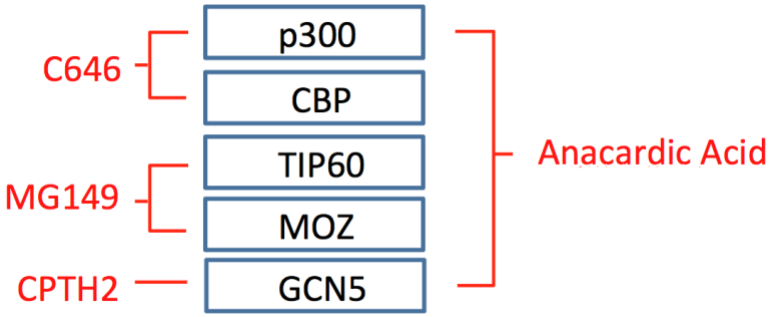


Figure 10. Targets of HAT inhibitors.
Anacardic acid was broadly used to inhibit all HATs to examine if this results in a decrease in acetylated SERCA2a. Small molecule inhibitors C646, MG149, and CPTH2 were then used to inhibit p300 and CBP, TIP60 and MOZ, and GCN5 respectively.



Statistical analysis

One-way analysis of variance between factors (ANOVA) and independent t-tests were used to detect differences between groups in objective 1. For objective 2, control versus HDAC inhibitor was analyzed by student T-test for independent samples. For objective 3, a one-way ANOVA was used for between-group comparisons of control, high glucose, and high glucose + HAT inhibitor. SERCA activity assays were analyzed by one-way ANOVA for between group comparisons. When significant differences ($p < 0.05$) were identified by ANOVA, a Tukey post-hoc test was used to identify differences between specific means. A sample size calculation determined that n=5 would be required to detect a 20% difference between groups with 80% power using a two-tailed alpha of 0.05. Smaller sample sizes were needed to detect larger differences between groups.

Chapter 3: Results

Objective 1 – Effect of high glucose on SERCA2a acetylation

To determine whether high glucose modulates acetylation of SERCA2a, we exposed rat primary cardiomyocytes to high glucose conditions for up 18 hours. All cells were harvested after 18 hours. Western blotting techniques looking at the 110 kDa band revealed that there was a 30% increase in acetylation at 4 hours, which was maintained up to 18 hours of exposure (**Figure 11**). Immunoprecipitation with acetylated lysine was used to detect the acetylation status of PGC-1α as a positive control for the high glucose conditions, [174]. High glucose resulted in a two-fold increase in PGC-1α acetylation (**Figure 12**). Immunoprecipitation was then used to confirm that the increase in acetyl-lysine at the 110 kDa band was SERCA2a-acetylation, which revealed a 37% increase in acetylated SERCA2a (**Figure 13**). SERCA2a assays were used to determine calcium dependent SERCA2a activity of samples. High glucose significantly impaired SERCA2a function by decreasing V_{max} by 38% and Ca_{50} by 25% (**Figure 14**).

Figure 11. Acetylated-lysine at 110kDa from rat primary cardiomyocytes treated with high glucose.
(A) A representative Western blot performed with acetylated lysine and β-actin antibodies on cardiomyocyte lysate. (B) Graphical representation of A demonstrating that high glucose (25 mM) exposure for 4 and 18 hours is capable of increasing acetylation of SERCA2a in adult rat primary cardiomyocytes. Acetylation calculated A-L/actin, experimental conditions reported relative to control. * different from Control ($P<0.05$). #, different from 1h ($P<0.05$). &, different from 2h ($P<0.05$). Graph represents the mean ± SEM (n=2).

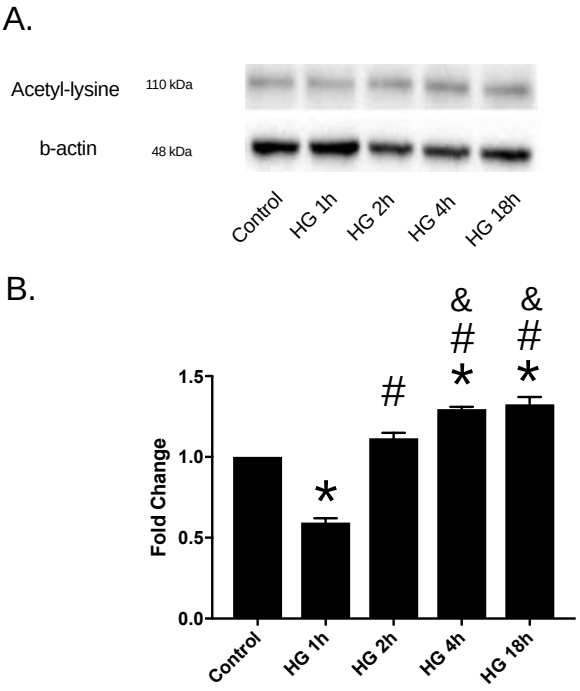


Figure 12. Acetylated PGC-1α in rat primary cardiomyocytes treated with high glucose. (A) Representative blot performed with PGC-1α antibody on IP samples followed by acetylated lysine antibody which was performed after stripping. (B) Graphical representation of A demonstrating that high glucose (25 mM) exposure for 18 hours is capable of increasing acetylation of PGC-1α in adult rat primary cardiomyocytes. Acetylation calculated as PGC-1α /A-L, high glucose reported relative to control. * different from Control ($P<0.05$). Graph represents the mean ± SEM (n=3).

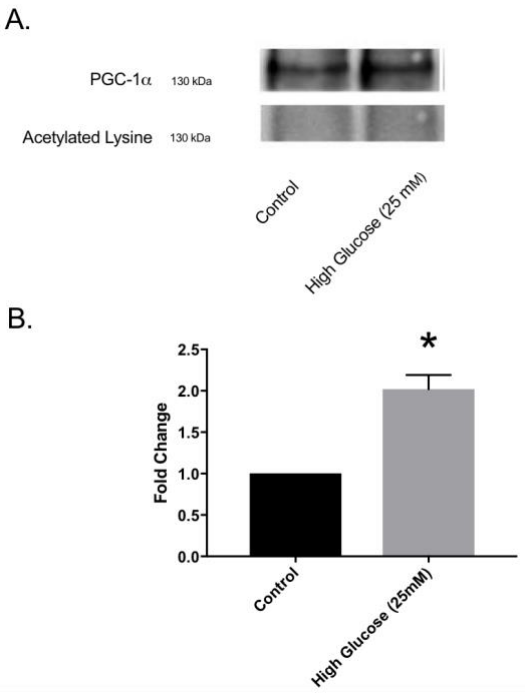


Figure 13. Acetylated SERCA2a in rat primary cardiomyocytes treated with high glucose.
(A) Representative blot performed with acetylated lysine antibody on IP samples followed by SERCA2a antibody which was performed after stripping. (B) Graphical representation of A demonstrating that high glucose (25 mM) exposure for 18 hours is capable of increasing acetylation of SERCA2a in adult rat primary cardiomyocytes. Acetylation calculated as A-L/SERCA2a, high glucose reported relative to control. * different from Control ($P<0.05$). Graph represents the mean ± SEM (n=6).

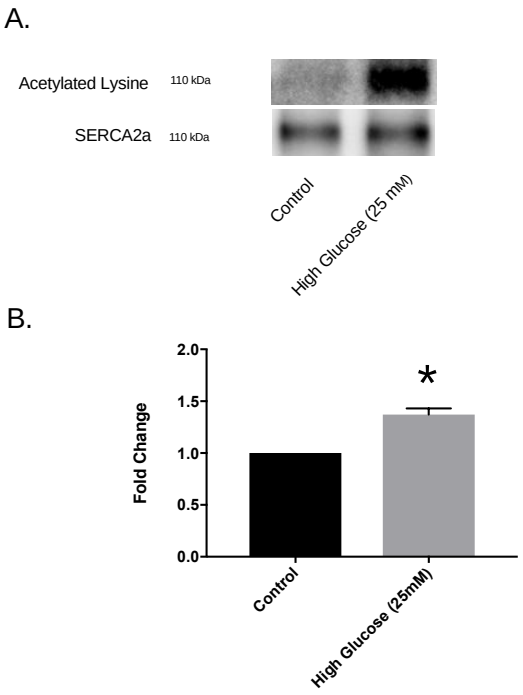
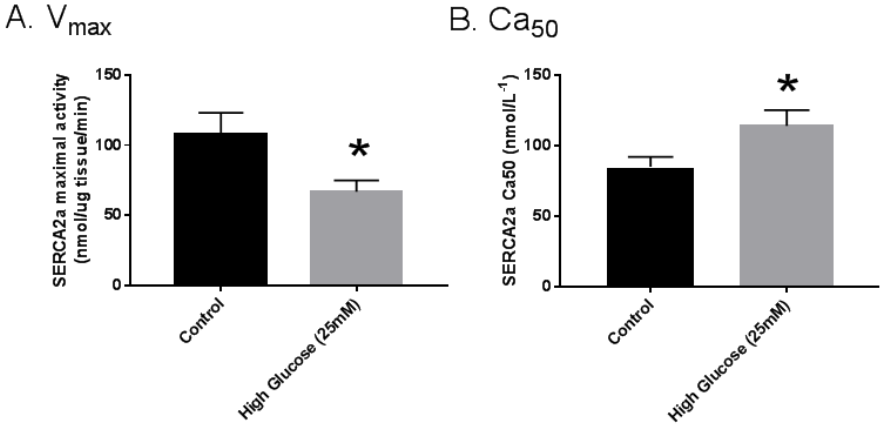


Figure 14. Calcium-dependent SERCA2a activity from rat primary cardiomyocytes treated with high glucose.
(A) Maximal SERCA2a activity (V_{max}) and (B) Calcium sensitivity, Ca_{50}. *, different from Control ($P<0.05$). Graph represents the mean ± SEM (n=5).



Objective 2 – HDAC inhibition

To identify the class of HDACs controlling acetylation of SERCA2a, cardiomyocytes were exposed to different small molecule inhibitors of the HDACs for 18 hours. Nicotinamide was used to inhibit the Class III HDACs (Sirtuins). Immunoprecipitation with acetylated lysine was used to examine the acetylation status of PGC-1α as a positive control, since it is known to be hyperacetylated when exposed to nicotinamide [174]. PGC-1α acetylation increased by 70% **(Figure 15**). The results of the immunoprecipitation experiments indicate an increase in SERCA2a acetylation by 36% (**Figure 16**). SERCA2a assays were used to determine calcium dependent SERCA2a activity of samples. Nicotinamide significantly impaired SERCA2a function by decreasing V_{max} by 33%. Ca_{50} was unchanged (**Figure 17**).

Figure 15. Acetylated PGC-1α in rat primary cardiomyocytes treated with nicotinamide. (A) Representative blot performed with PGC-1α antibody on IP samples followed by acetylated lysine antibody which was performed after stripping. (B) Graphical representation of A demonstrating that nicotinamide (10 mM) exposure for 18 hours is capable of increasing acetylation of PGC-1α in adult rat primary cardiomyocytes. Acetylation calculated as PGC-1α /A-L, nicotinamide condition reported relative to control. * different from Control ($P<0.05$). Graph represents the mean ± SEM (n=3).

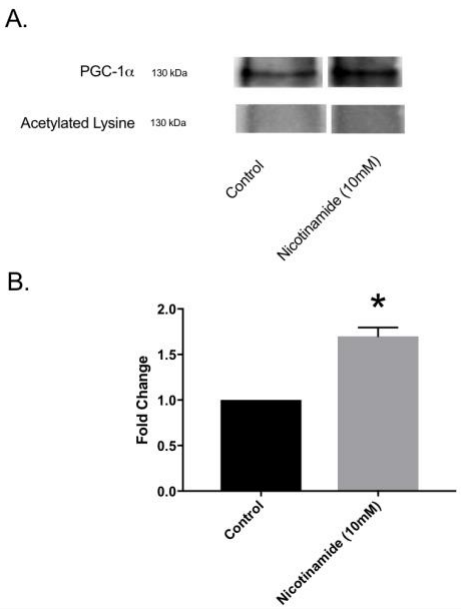


Figure 16. Acetylated SERCA2a in rat primary cardiomyocytes treated with nicotinamide.
(A) A representative blot performed with acetylated lysine antibody on IP samples followed by SERCA2a antibody performed after stripping. (B) Graphical representation of A demonstrating that nicotinamide (10 mM) exposure for 18 hours is capable of increasing acetylation of SERCA2a in adult rat primary cardiomyocytes. Acetylation calculated as A-L/SERCA2a, nicotinamide condition reported relative to control. *, different from Control ($P<0.05$). Graph represents the mean ± SEM (n=5).

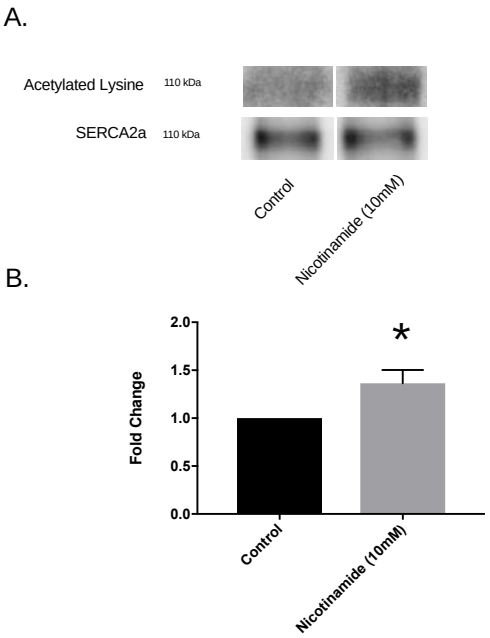
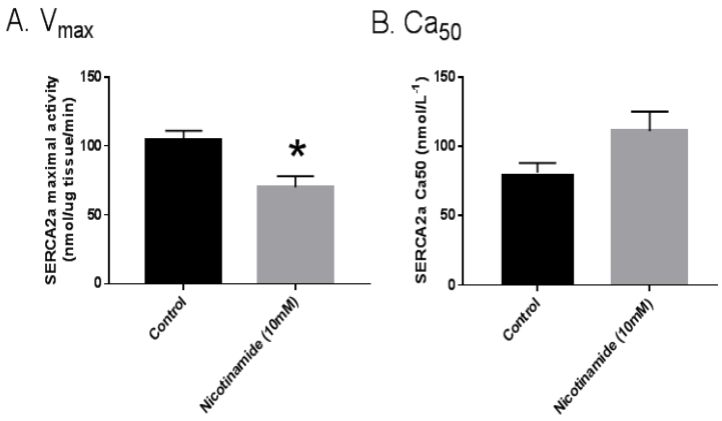


Figure 17. Calcium-dependent SERCA2a activity from rat primary cardiomyocytes treated with nicotinamide.
(A) Maximal SERCA2a activity (V_{max}) and (B) Calcium sensitivity, Ca_{50}. *, different from Control ($P<0.05$). Graph represents the mean ± SEM (n=5).



Trichostatin A was used to inhibit Class I and Class II HDACs to examine if non-sirtuin HDACs modulate SERCA2a acetylation. Western blot with Acetylated Histone H3 K9/K14 antibody was used as a positive control for 1μM Trichostatin A. Acetylation of histone H3 K9/K14 increased 27-fold with Trichostatin A (**Figure 18**). Results of immunoprecipitation indicated no increase in SERCA2a acetylation with inhibition of Class I and Class II (non-sirtuin) HDACs using Trichostatin A (**Figure 19**). Trichostatin A did not significantly alter SERCA2a function (**Figure 20**).

Figure 18. Acetyl-histone H3 (K9/14) in rat primary cardiomyocytes treated with TSA. **(**A) A representative Western blot performed with acetyl-histone H3 (K9/K14) and β-actin antibodies on cardiomyocyte lysate. (B) Graphical representation of A demonstrating that TSA (1 μM) exposure for 18 hours is capable of increasing acetylation of histone H3 K9/14 in adult rat primary cardiomyocytes. Acetylation calculated as Acetyl-H3(K9/K14)/ β-actin, TSA condition reported relative to control. *, different from Control ($P<0.05$). Graph represents the mean ± SEM (n=2).

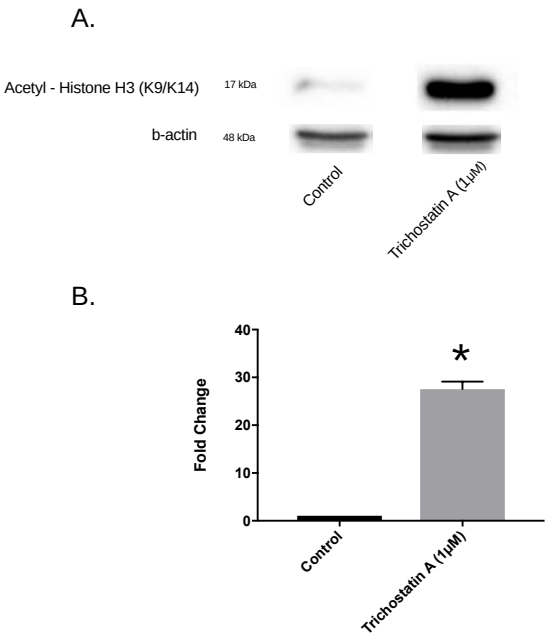


Figure 19. Acetylated SERCA2a in rat primary cardiomyocytes treated with TSA.
(A) A representative blot performed with acetylated lysine antibody on IP samples followed by SERCA2a antibody performed after stripping. (B) Graphical representation of A demonstrating that TSA (1 μM) exposure for 18 hours did not increase acetylation of SERCA2a in adult rat primary cardiomyocytes. Acetylation calculated as A-L/SERCA2a, TSA condition reported relative to control. *, different from Control ($P<0.05$). Graph represents the mean ± SEM (n=5).

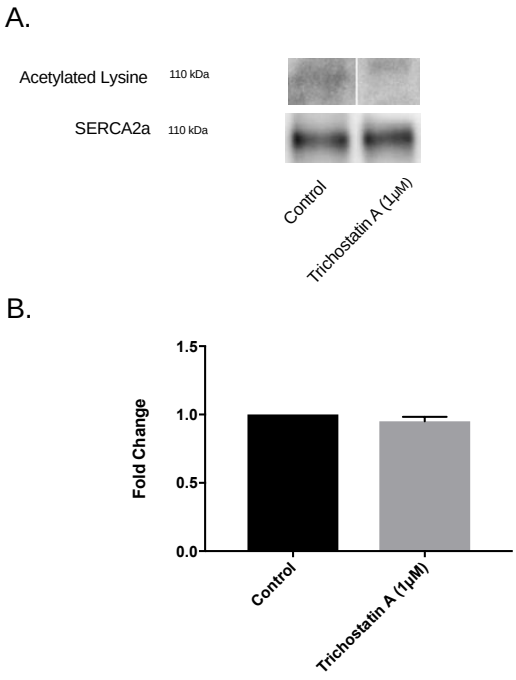
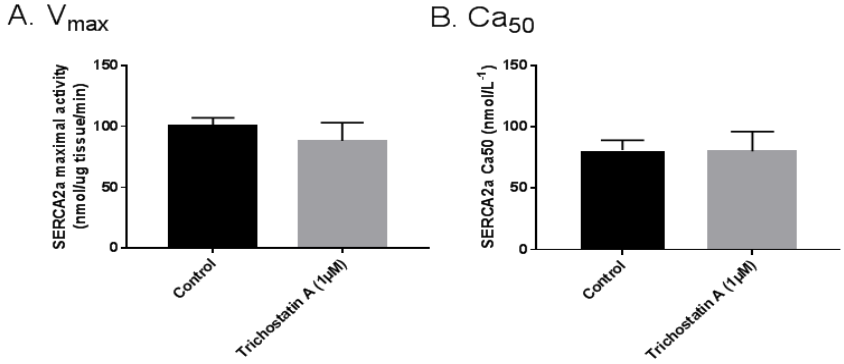


Figure 20. Calcium-dependent SERCA2a activity from rat primary cardiomyocytes treated with TSA.
(A) Maximal SERCA2a activity (V_{max}) and (B) Calcium sensitivity, Ca_{50}. Graph represents the mean ± SEM (n=5).



Objective 3 – Effect of HATs on glucose induced SERCA2a acetylation

To identify HATs that are responsible for acetylation of SERCA2a, cardiomyocytes were exposed to small molecule inhibitors of the HATs for 1 hour prior to glucose exposure to determine which HATs were responsible for the high glucose induced acetylation. Cardiomyocytes were pretreated with anacardic acid to determine if broad inhibition of all HATs could prevent the increase in SERCA2a acetylation induced by glucose. Immunoprecipitation with acetylated lysine was used to examine the acetylation status of PGC-1α, which has been shown to be acetylated by GCN5 as a positive control for HAT inhibition by anacardic acid [174]. PGC-1α acetylation did not differ from control when pre-treated with anacardic acid prior to high glucose **(Figure 21)**. One hour of pre-treatment with anacardic acid attenuated glucose induced SERCA2a acetylation (**Figure 22)**. However, anacardic acid was not able to fully rescue the decrease V_{max} and Ca_{50} caused by high glucose treatment (**Figure 23**).

Figure 21. Acetylated PGC-1α in rat primary cardiomyocytes treated with anacardic acid and high glucose.
(A) Representative blot performed with PGC-1α antibody on IP samples followed by acetylated lysine antibody which was performed after stripping. (B) Graphical representation of A demonstrating that anacardic acid (3μM) exposure for 1 hour prior to 18 hours of high glucose can prevent the increase in acetylation of PGC-1α caused by high glucose in adult rat primary cardiomyocytes. Acetylation calculated as PGC-1α /A-L, experimental condition reported relative to control. * different from Control ($P<0.05$). #, different from High Glucose ($P<0.05$). Graph represents the mean ± SEM (n=3).

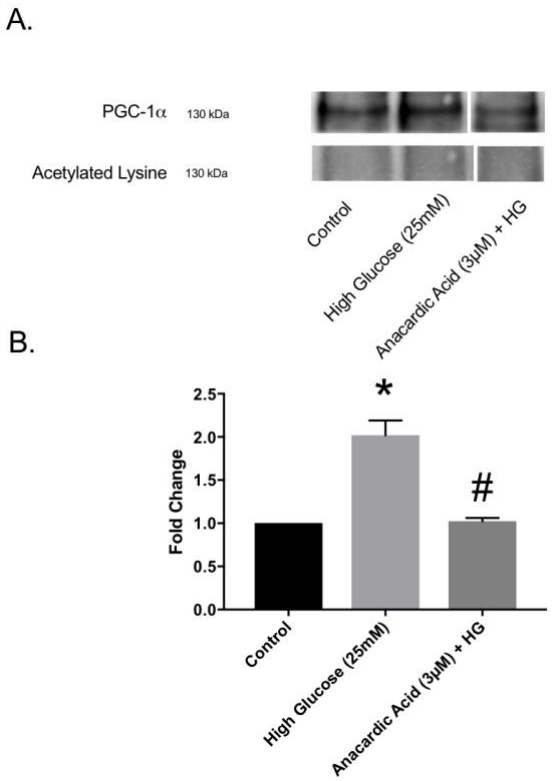


Figure 22. Acetylated SERCA2a in rat primary cardiomyocytes treated with anacardic acid and high glucose.
(A) A representative blot performed with acetylated lysine antibody on IP samples followed by SERCA2a antibody performed after stripping. (B) Graphical representation of A demonstrating that anacardic Acid (3 μM) exposure for 1 hour prior to and during 18 hours of high glucose exposure can prevent glucose induced acetylation of SERCA2a in adult rat primary cardiomyocytes. Graph represents the mean ± SEM (n=4) that anacardic Acid attenuated the glucose induced increase in SERCA2a acetylation. Acetylation calculated as A-L/SERCA2a, experimental conditions reported relative to control. *, different from Control (P<0.05). #, different from high glucose (P<0.05).

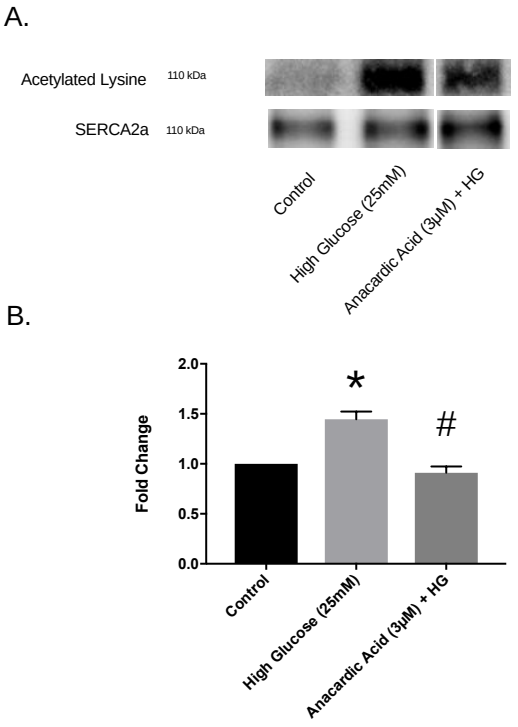
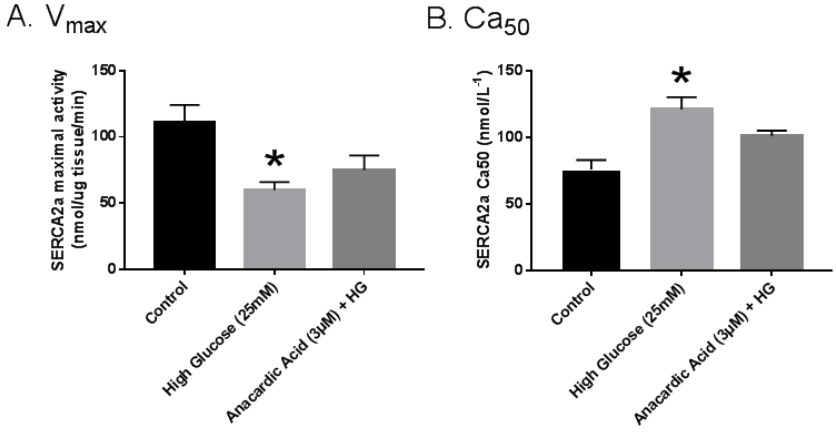


Figure 23. Calcium-dependent SERCA2a activity from rat primary cardiomyocytes treated with anacardic acid and high glucose.
(A) Maximal SERCA2a activity (V_{max}) and (B) Calcium sensitivity, Ca_{50}. *, different from Control (P<0.05). Graph represents the mean ± SEM (n=4).



C646 was used to determine if p300/CBP inhibition could prevent the increase in SERCA2a acetylation induced by glucose. Immunoprecipitation with acetylated lysine was used to assess the acetylation status of PGC-1α as p300 has been shown to acetylate it [184]. PGC-1α acetylation did not differ from control when pre-treated with C646 prior to high glucose treatment (**Figure 24**). No change in SERCA2a acetylation was observed with pre-treatment using C646 compared to high glucose (**Figure 25**). As no change in acetylation was observed with C646 treatment, SERCA2a activity assays were not performed for C646 samples.

Figure 24. Acetylated PGC-1α in rat primary cardiomyocytes treated with C646 and high glucose.
(A) Representative blot performed with PGC-1α antibody on IP samples followed by acetylated lysine antibody which was performed after stripping. (B) Graphical representation of A demonstrating that C646 (5μM) exposure for 1 hour prior to 18 hours of high glucose can prevent the increase in acetylation of PGC-1α caused by high glucose in adult rat primary cardiomyocytes. Acetylation calculated as PGC-1α /A-L, experimental condition reported relative to control. * different from Control (P<0.05). #, different from high glucose (P<0.05). Graph represents the mean ± SEM (n=2).

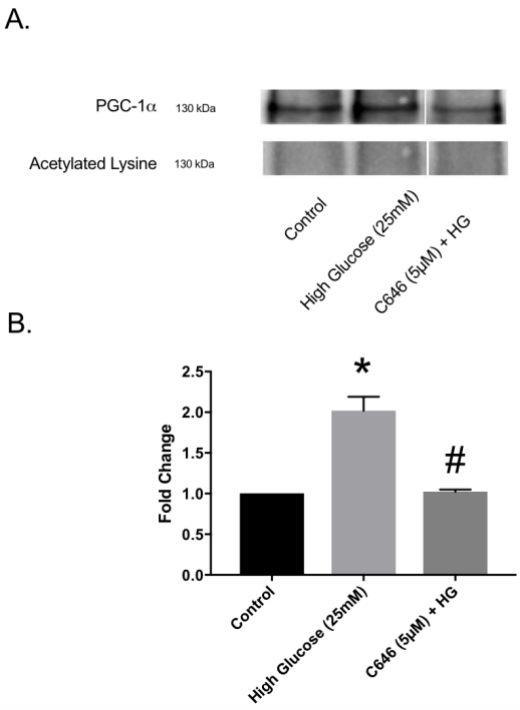
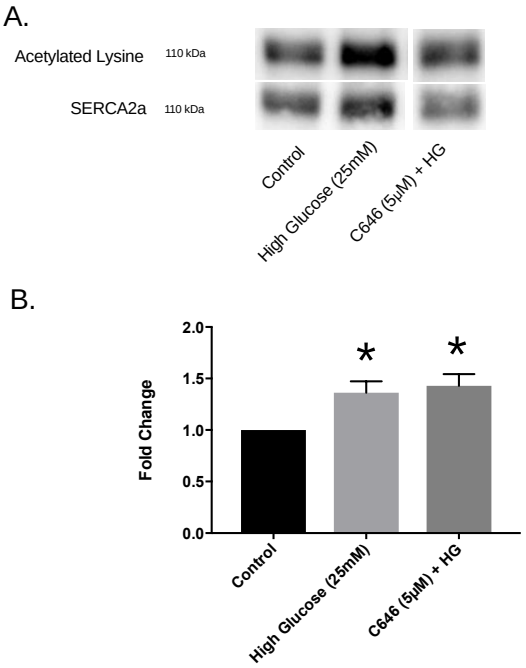


Figure 25. Acetylated SERCA2a in rat primary cardiomyocytes treated with C646 and high glucose.
(A) A representative blot performed with acetylated lysine antibody on IP samples followed by SERCA2a antibody performed after stripping. (B) Graphical representation of A demonstrating that C646 (5 μM) exposure for 1 hour prior to and during 18 hours of high glucose exposure does not prevent glucose induced acetylation of SERCA2a in adult rat primary cardiomyocytes. Graph represents the mean ± SEM (n=4) that C646 did not reduce the glucose induced increase in SERCA2a acetylation. Acetylation calculated as A-L/SERCA2a, experimental conditions reported relative to control. *, different from Control ($P<0.05$).



MG149 was used to determine if inhibition TIP60 and MOZ could prevent the increase in SERCA2a acetylation induced by glucose. Several concentrations of MG149 were used with the goal of TIP60/MOZ inhibition, however all concentrations resulted in a significant reduction in cell viability. Due to the reduction in cell viability with MG149 treatment SERCA2a activity assays were not performed on MG149 samples. While a formal viability assay was not performed, in future work calcein-AM and ethidium

homodimer-1 could be used to visualize the number of live and dead cells [198,199]. In a paper released after the design of this project, the authors describe a significant reduction in cell viability of precision cut lung slices above a threshold concentration of MG149, which corroborates our results [199].

CPTH2 was used to determine if inhibition of GCN5 could prevent the increase in SERCA2a acetylation induced by glucose. Immunoprecipitation with acetylated lysine was used to assess the acetylation status of PGC-1α as GCN5 is known to acetylate PGC-1α [174]. PGC-1α acetylation was not different from control when pre-treated with CPTH2 prior to high glucose (**Figure 26**). One hour of pre-treatment with CPTH2 resulted in attenuation of glucose induced SERCA2a acetylation (**Figure 27)**. However, CPTH2 was not able to fully reverse the decrease V_{max} and Ca_{50} caused by high glucose (**Figure 28**). As there was a similar effect size to that seen with anacardic acid, we did not try a condition with all 3 specific inhibitors.

Figure 26. Acetylated PGC-1α in rat primary cardiomyocytes treated with CPTH2 and high glucose.

(A) Representative blot performed with PGC-1α antibody on IP samples followed by acetylated lysine antibody which was performed after stripping. (B) Graphical representation of A demonstrating that CPTH2 (10μM) exposure for 1 hour prior to 18 hours of high glucose can prevent the increase in acetylation of PGC-1α caused by high glucose in adult rat primary cardiomyocytes. Acetylation calculated as PGC-1α /A-L, experimental condition reported relative to control. * different from Control (P<0.05). #, different from high glucose (P<0.05). Graph represents the mean ± SEM (n=3).

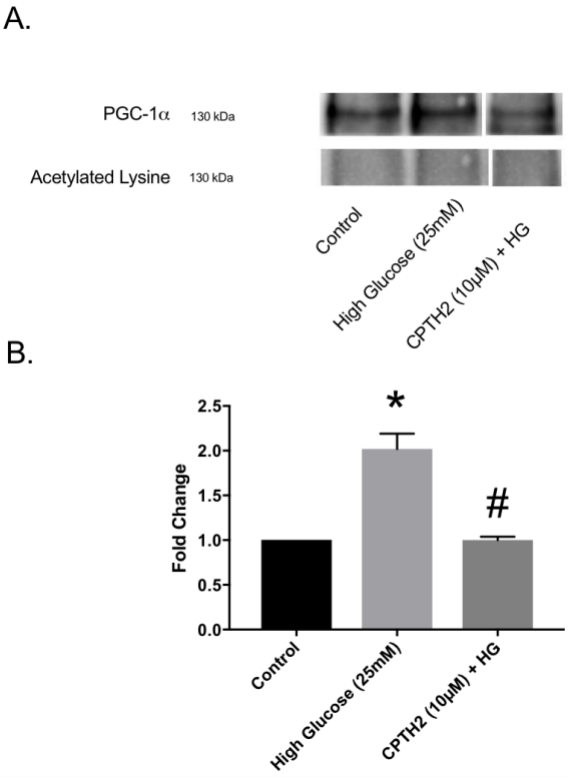


Figure 27. Acetylated SERCA2a in rat primary cardiomyocytes treated with CPTH2 and high glucose.

(A) A representative blot performed with acetylated lysine antibody on IP samples followed by SERCA2a antibody performed after stripping. (B) Graphical representation of A demonstrating that CPTH2 (10 μM) exposure for 1 hour prior to and during 18 hours of high glucose exposure can prevent glucose induced acetylation of SERCA2a in adult rat primary cardiomyocytes. Graph represents the mean ± SEM (n=6) that CPTH2 attenuated the glucose induced increase in SERCA2a acetylation. Acetylation calculated as A-L/SERCA2a, experimental conditions reported relative to control. *, different from Control (P<0.05). #, different from high glucose (P<0.05).

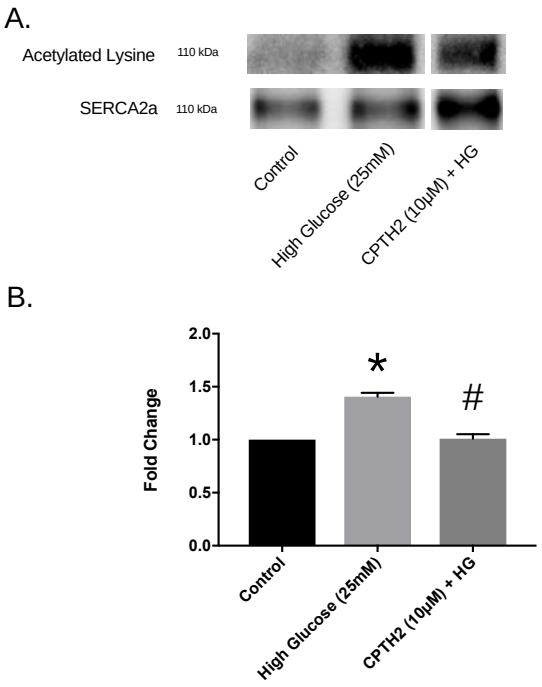
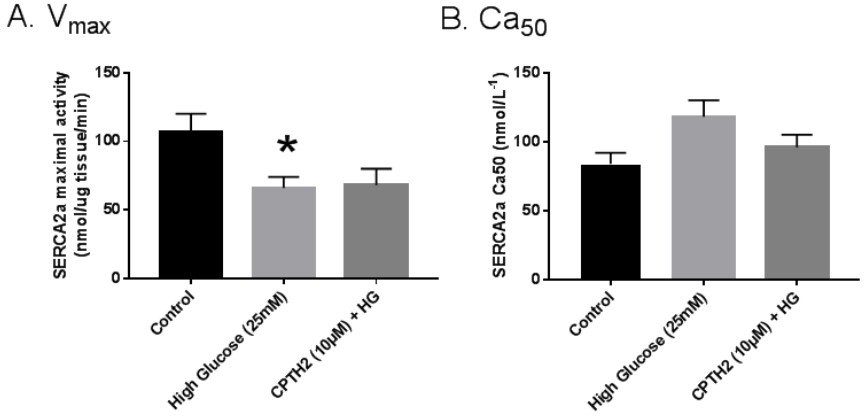


Figure 28. Calcium-dependent SERCA2a activity from rat primary cardiomyocytes treated with CPTH2 and high glucose.

(A) Maximal SERCA2a activity (V_{max}) and (B) Calcium sensitivity, Ca_{50}. *, different from Control (P<0.05). Graph represents the mean ± SEM (n=4).



Chapter 4: Discussion

It is known that SERCA2a can be acetylated at 3 sites, however, the enzymes that regulate this process have not yet been identified. Thus, the objectives of this study were to determine if high glucose exposure alters the acetylation level of SERCA2a (Objective 1), to identify the HDAC(s) responsible for the regulation of SERCA2a acetylation (Objective 2), and finally, to identify the HAT(s) responsible for the regulation of SERCA2a acetylation under high glucose conditions.

High glucose exposure increases SERCA2a acetylation

A high glucose state influences several mechanisms known to impair SERCA2a activity. These mechanisms include post-translational modifications and protein-protein interactions. High glucose treatment leads to nitration of SERCA, which decreases V_{max} by ~25% [113,114]. SERCA2a inhibition can also occur through alterations in expression levels of PLN or through decreased phosphorylation of PLN in the diabetic heart [117,200]. Increased interaction of SERCA2a with PLN has also been documented in streptozotocin injected rats, in which plasma glucose levels were high [85]. It has been demonstrated that mutation of two lysine residues within the nucleotide binding domain of SERCA2a, lysine^{480} and lysine^{585}, leading to a notable reduction in ATP binding affinity [201,202]. This effect occurs by preventing SUMOylation of SERCA2, and results in decreased SERCA2a function [201,202]. The work presented in this thesis is the first to determine that high glucose increases SERCA2a acetylation in primary cardiomyocytes. It is unclear why SERCA2a acetylation decreased after 1 hour of glucose exposure. This could be addressed using time-matched controls in future studies. When SERCA2a acetylation

was examined using immunoprecipitation **(Figure 13)**, we determined that SERCA2a acetylation was increased by 37% compared to control when incubated in 25 mM glucose media. This finding suggests that SERCA2a acetylation is indeed enhanced in cardiomyocytes when exposed to high glucose conditions in cell culture. Assessment of SERCA2a activity determined that high glucose significantly impaired SERCA2a function by decreasing V_{max} by 38% and Ca_{50} by 25%. That observation supports previous work by our group which demonstrated a 32% decrease in the V_{max} of SERCA2a in low-dose streptozotocin injected mice fed a high fat diet. The decrease in V_{max} was accompanied by a 32% decrease in SERCA2a protein content [49]. This thesis provides data that suggests acetylation as a novel mechanism contributing to impairment of SERCA2a activity in a high glucose state. Lysine 464, lysine 510, and lysine 533 of SERCA2a all reside within the cytoplasmic nucleotide binding domain and can be acetylated [203,204]. Recent data from our lab demonstrated that a SERCA2a mutant which mimics constitutively acetylated at all 3 acetylation sites, resulted in a 30% decrease in V_{max} compared to an acetylation deficient mutant [135]. This supports the idea that the increased acetylation within the nucleotide binding domain of SERCA2a results in a corresponding decrease in V_{max} due to a change in ATP binding affinity. However, the acetylation profile induced by high glucose remains unclear. It is still unknown whether high glucose causes acetylation at a specific acetylation site and if each site plays a role in decreasing SERCA2a $V_{max.}$ In addition to this, it is unclear as to why acetylation does not increase to a greater extent than 37% in high glucose. This is likely accounted for by the fact that only a subset of total SERCA2a is acetylated in the cell. Further research will need to be conducted to further elucidate the localization of SERCA2a acetylation.

High glucose treatment also increased the Ca_{50} of SERCA2a. This change was unexpected. Ca_{50} is a measure of calcium sensitivity and the primary mechanism through which Ca_{50} is altered is by binding with PLN. Thus, the decrease in Ca_{50} may be due to increased inhibitory activity by PLN, which is known to decrease the calcium sensitivity of SERCA in the presence of high glucose [85]. Due to the length of glucose treatment, it is possible that this happened through a decrease in phosphorylated PLN (p-PLN) rather than a change in PLN protein level [119]. However, we did not measure PLN or p-PLN and thus, cannot make any definitive conclusions on the role of PLN. PLN phosphorylation was not measured because previous work from our lab using Cos-1 mutants expressing constitutively acetylated SERCA2a demonstrated no change in calcium sensitivity as compared to control cells. This data is further supported by the fact that Cos-1 cells do not express, thus demonstrating the key role of PLN in modulating calcium sensitivity and that the acetylation induced changes in V_{max} are independent of this [203,204]. Furthermore, it is unlikely that SERCA2a acetylation alters the inhibitory action of PLN as the acetylation sites of SERCA2a are not in close proximity to the residues of SERCA2a with which PLN interacts [203,204]. It is also possible that post-translational modifications of SERCA2a other than acetylation occurred to cause the change in calcium sensitivity. The pleiotropic effects of high glucose exposure are one of the limitations of this model in examining post-translational modifications. Thus, we also used chemical inhibition of HDACs and HATs to determine the pathways involved with SERCA2a acetylation by targeting them more directly.

SERCA2a activity has been shown to be regulated by multiple different post-translational modifications including glutathionylation [99], nitration [100], glycosylation [101],

O-GlnNAcylation [102], SUMOlyation [103,104], and acetylation [103,105]. All of these can be influenced by high glucose and thus, further research must be conducted to elucidate the specific roles and conditions of each modification. Additionally, this research should examine different combinations of post-translational modifications, as there is some evidence that post-translational modifications may influence one another. It has been suggested that SUMOlyation increases ATP binding affinity and that this may occur through antagonism to acetylation [202]. However, we do not know if other post-translational modifications interact to increase or decrease SERCA2a acetylation. We made the decision not to measure all the post-translational modifications of SERCA2a that occur in the high glucose model because the primary goal of this project was to confirm if high glucose can increase acetylation of SERCA2a. Therefore, we used alternative methods to increase acetylation to corroborate the acetylation changes that occurred in the high glucose condition.

Sirtuins are involved in the regulation of SERCA2a acetylation

Diabetes and aging are both major risk factors for cardiovascular disease and both have also been shown to decrease sirtuin function [168,205]. In addition to this, NAD^+, the substrate for the sirtuins, is reduced in cardiac disease [206,207]. Together, this suggests that a multifactorial decrease in sirtuin activity might play a role in the pathophysiology of diabetic heart disease. Current data suggests that aggressive glycemic control does not significantly reduce macrovascular complications in patients with established cardiovascular disease. However, for patients with no macrovascular disease, tighter glycemic control is recommended. It is possible that this group of patients are the ones

who might benefit most from SERCA2a therapy, as calcium cycling is one of the earliest changes that occurs in diabetic cardiomyopathy [21].

Inhibition of sirtuins increases SERCA2a acetylation

Due to the relationship between the sirtuins, diabetes, and cardiovascular disease, we examined the role of sirtuins deacetylases on the acetylation status of SERCA2a as a possible mechanism for the increased SERCA2a acetylation seen in high glucose conditions. When the sirtuin family (Class III HDACs) were inhibited by using 10 mM nicotinamide, we observed a 36% increase in acetylation of SERCA2a (**Figure 16**). Assessment of SERCA2a activity demonstrated a corresponding 33% decrease in V_{max}. This change matches with the change in acetylated SERCA2a observed when cardiomyocytes were treated with high glucose. Ca_{50} was unchanged with nicotinamide treatment but Ca_{50} was changed in the high glucose condition. This suggests that the inhibition of the sirtuins with nicotinamide might influence SERCA2a more specifically than treatment with high glucose. Nicotinamide acts by inhibiting the sirtuins, and as a result, it may have a more direct influence on acetylation than high glucose treatment [207]. Together, this data strongly suggests that the sirtuin family is involved in the deacetylation of SERCA2a, with impairment of the sirtuins leading to increased SERCA2a acetylation and decreased SERCA2a maximal activity.

Inhibition of GCN5 prevents the glucose-induced increase SERCA2a acetylation

When the HATs were broadly inhibited using anacardic acid for 1 hour prior to glucose exposure, the glucose-induced increase in acetylation was attenuated, suggesting that one of the HATs inhibited by anacardic acid is involved in the acetylation of

SERCA2a (**Figure 22**). This effect also occurred when GCN5 was inhibited which makes sense, as the actions of GCN5 have been shown to oppose that of the sirtuins [172,174,175]. Unexpectedly, assessment of SERCA2a activity determined that although broad inhibition of the HATs and GCN5 prevented SERCA2a acetylation in high glucose conditions, V_{max} and Ca_{50} was not rescued. The first reason for this disconnect is cell-stress or off target effects caused by the HAT inhibitors. A qualitative observation in our study was that when cells were treated with anacardic acid, they appeared to have decreased survival with more dead cells being present prior to harvesting. This suggests that at the concentration used, anacardic acid may have had a negative effect on other cellular processes resulting or may have potentially caused an alternative post-translation of SERCA2a. It has been documented that anacardic acid is an inducer of endoplasmic reticulum stress and this effect may account for the impaired SERCA2a activity in these samples [208]. An alternative explanation as to why the V_{max} did not improve is that glucose treatment resulted in an alternative post-translational modification which could impair calcium-cycling and that this change was not prevented by HAT inhibition. This could also be the reason for the lack of improvement in Ca_{50}, however it is more likely that this change is due to a change in p-PLN as previously described. Targeting GCN5 could play a crucial role in treating diabetic cardiomyopathy and heart failure. However, further research must be done to more specifically elucidate the role of GCN5 in SERCA2a acetylation and if inhibition of GCN5 can recover SERCA2a activity if it is more directly targeted.

While this data suggests that the sirtuin family is involved in SERCA2a deacetylation, it does not identify which sirtuin is specifically responsible for the changes

observed. Due to the cellular locations of the sirtuins, SIRT1-3 are the most likely to be involved as all of them have been detected in the cytoplasm [209–211]. The beneficial of effect of sirtuin activity on SERCA2a function has been demonstrated using resveratrol, a SIRT1 activator. Resveratrol treatment resulted in upregulation SERCA2a and improved cardiac function [205]. While that study did not look at acetylation, based on our data that demonstrates a role of the sirtuins on SERCA2a acetylation, it is possible that some of the improvements in cardiac function occurred due to decreased SERCA2a acetylation in addition to the increased SERCA2a content. It is currently unknown whether SERCA2a is deacetylated directly by the sirtuins or if they deacetylate another cytosolic HDAC which subsequently deacetylates SERCA2a. Additionally, there are several other proteins that are involved in SERCA2a activity regulation. Thus, it is possible that the sirtuins influence these proteins which then modify SERCA2a activity. One example is AMPK, which can increase expression and activity of SERCA2a and is known to be activated through deacetylation by SIRT3 [212,213]. Thus, further experiments are needed to determine the specific role and sirtuin involved in the regulation of SERCA2a acetylation. This data demonstrates the role of the sirtuins in the deacetylation of SERCA2a. Due to this, therapies targeting sirtuin activity or protein levels could potentially be effective in treating diabetic cardiomyopathy or heart failure when there are high levels of acetylated SERCA2a.

Non-sirtuin pathways are unlikely to be involved in SERCA2a acetylation

To examine the role of non-sirtuin HDACs, we inhibited Class I and Class II HDACs using Trichostatin A. This did not result in any increase of SERCA2a acetylation (**Figure 19**). Additionally, V_{max} did not change with inhibition of Class I and Class II HDACs. Ca_{50} was unchanged in both Trichostatin A and Nicotinamide treatment. Class I HDACs are located primarily in the nucleus and because of this, it is unsurprising that they do not act on the cytoplasmic acetylation sites of SERCA2a [214]. Class I HDACs are capable of shuttling between nucleus and cytoplasm based on phosphorylation status, however our data suggests that even with this ability they are not involved in SERCA2a acetylation [214]. However, even without acting on SERCA2a, HDACs have been shown to play a role in the heart. HDAC inhibition with TSA has been shown to prevent pathological hypertrophy in a transverse aortic constriction model of pressure overload [215]. Thus, although Class I and II HDACs do not appear to act on SERCA2a acetylation, targeting them under different circumstances may be beneficial.

Implications and future directions

Together, this thesis provides preliminary data that may have implications for the use of SERCA2a acetylation strategies in the context of diabetes-induced heart disease, where glucose levels are high. One approach to management would be to prevent the acetylation of SERCA2a by blocking the acetylation sites on the SERCA2a protein. Alternatively, increasing the activity of the sirtuins or decreasing GCN5 activity could be used to alter the level of SERCA2a acetylation in the heart. This may help prevent the negative changes to calcium cycling and impairment of SERCA2a that occurs in the

diabetic heart. These techniques could likely be accomplished through pharmacological methods. Due to the success of CUPID 1, targeting SERCA2a in humans is a valid strategy to improve cardiac function. However, even though a percutaneous intervention is less invasive than surgery, it is still considered an invasive procedure. By using pharmacological approaches to improve SERCA2a activity it may be possible to avoid the risks associated with these types of procedures.

To confirm the findings of this thesis, further studies should be performed. One aspect of this would be to further characterize the mechanism of SERCA2a acetylation on activity and to see if specific acetylation sites have different effects of SERCA2a activity. Additionally, a FITC assay could be employed to identify structural changes in the ATP binding domain and if this is indeed the mechanism through which acetylation alters activity. In addition to this, cell culture experiments with mutants that mimic acetylation at single and double sites should be performed to assess if there is a specific acetylation site responsible for the changes seen when SERCA2a is acetylated. Experiments to assess whether there is a critical concentration of glucose at which SERCA2a acetylation increases should be performed by exposing cells to various levels of glucose and assessing acetylation. Additionally, the role of SIRTs could be solidified through gain and loss experiments. Upregulation of SIRTs would be expected to decrease SERCA2a acetylation with downregulation of SIRTs increasing SERCA2a acetylation. Finally, an *in vivo* using mice overexpressing SIRTs and mice under expressing SIRTs could be exposed to diabetogenic conditions to compare changes in SERCA2a acetylation and the resulting cardiac function.

Study Limitations

One of the limitations of our study is the use of chemical inhibitors to influence post-translational modifications. While the inhibitors we used have been shown to affect the HATs and HDACs that they were used to examine, it is unknown if they have other off target effects that may have influenced SERCA2a activity or cell viability. This decrease in viability was particularly evident in the cells treated with MG149, which were excluded due to decreased cell survival in comparison to other conditions. A similar decrease in viability was described above a threshold concentration of ~10μM MG149 in another study using precision-cut lung slices that was released after our study had finished [199]. Although that concentration was higher than the concentrations used in our study, it is possible that the cardiomyocytes have a higher sensitivity to the compound and resulted in a similar effect. This demonstrates one of the limitations of using chemical inhibitors. However, as this is preliminary research in the area, the methods are appropriate. Future experiments could use shRNA or adenoviral transfection to decrease or increase protein levels of the targets we have identified in this study. A second limitation of the experiment was the high glucose model. While this model is a commonly used model to mimic a diabetic state, high glucose has many other effects on the cell. Due to this, we cannot exclude the role of other post-translational modifications of SERCA2a on the changes in activity we saw. Further studies are needed to better clarify the conditions and time courses of glucose exposure that influence various post-translational modifications of SERCA2a. Another study limitation is that SERCA2a protein content was not directly measured. However, as the half-life of SERCA2a protein is 4.9 days [202], it is unlikely that SERCA2a concentration changed between conditions.

The final limitation of our study is that it is a cell culture model. This model is commonly used to examine signaling pathways, however, generalizability is always a limitation. Therefore, animal studies using transgenic mice to examine the sirtuins and GCN5 as we have identified in this study could be used to better demonstrate the role of these proteins *in vivo*.

Conclusions

Our study is novel because it is the first to demonstrate that high glucose results in increased acetylation of SERCA2a with a corresponding decrease in SERCA2a activity. This demonstrates one of the ways in which high glucose levels might result in impaired calcium cycling in the diabetic heart. Furthermore, we have shown that inhibition of the sirtuins is able to increase the acetylation of SERCA2a and decrease maximal SERCA2a activity. As the sirtuins are also impaired by high glucose, this data helps us to better understand the pathophysiology of the impaired calcium-cycling that occurs in a high glucose state which may also occur in diabetes. As the sirtuins are activated by both dietary and exercise interventions this also helps to illustrate the mechanism as to why these interventions have a positive effect on cardiovascular health. Finally, we demonstrated that broad inhibition of the HATs as well as specific inhibition of GCN5 results in a decrease in SERCA2a acetylation. Together, this data will help to inform future work to further our understanding of the role of post-translational modifications of SERCA2a in the heart and develop strategies targeting these post-translational modifications to better treat patients with diabetic cardiomyopathy and heart failure.

Reference List

1. Government of Canada, P. H. A. of C. Diabetes in Canada: Facts and figures from a public health perspective - Public Health Agency of Canada. (2011). Available at: http://www.phac-aspc.gc.ca/cd-mc/publications/diabetes-diabete/facts-figures-faits-chiffres-2011/index-eng.php. (Accessed: 7th June 2017)

2. Grundy, S. M. *et al.* Diabetes and Cardiovascular Disease. *Circulation* **100,** 1134–1146 (1999).

3. Aneja, A., Tang, W. H. W., Bansilal, S., Garcia, M. J. & Farkouh, M. E. Diabetic Cardiomyopathy: Insights into Pathogenesis, Diagnostic Challenges, and Therapeutic Options. *Am. J. Med.* **121,** 748–757 (2008).

4. Stratton, I. M. *et al.* Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* **321,** 405–412 (2000).

5. Terry, T., Raravikar, K., Chokrungvaranon, N. & Reaven, P. D. Does Aggressive Glycemic Control Benefit Macrovascular and Microvascular Disease in Type 2 Diabetes?: Insights from ACCORD, ADVANCE, and VADT. *Curr. Cardiol. Rep.* **14,** 79–88 (2012).

6. Doenst, T., Nguyen, T. D. & Abel, E. D. Cardiac Metabolism in Heart Failure - Implications beyond ATP production. *Circ. Res.* **113,** 709 (2013).

7. Kolwicz, S. C., Purohit, S. & Tian, R. Cardiac Metabolism and its Interactions With Contraction, Growth, and Survival of Cardiomyocytes. *Circ. Res.* **113,** 603–616 (2013).

8. Rodrigues, B., Cam, M. C. & McNeill, J. H. Metabolic disturbances in diabetic

cardiomyopathy. *Mol. Cell. Biochem.* **180,** 53–57 (1998).

9. Shao, D. & Tian, R. Glucose Transporters in Cardiac Metabolism and Hypertrophy. *Compr. Physiol.* **6,** 331–351 (2015).

10. Boudina, S. & Abel, E. D. Diabetic Cardiomyopathy Revisited. *Circulation* **115,** 3213–3223 (2007).

11. Neubauer, S. *et al.* Myocardial Phosphocreatine-to-ATP Ratio Is a Predictor of Mortality in Patients With Dilated Cardiomyopathy. *Circulation* **96,** 2190–2196 (1997).

12. Neubauer, S. The failing heart--an engine out of fuel. *N. Engl. J. Med.* **356,** 1140–1151 (2007).

13. Fukushima, A. & Lopaschuk, G. D. Acetylation control of cardiac fatty acid β-oxidation and energy metabolism in obesity, diabetes, and heart failure. *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* **1862,** 2211–2220 (2016).

14. Pinz, I. *et al.* Compromised myocardial energetics in hypertrophied mouse hearts diminish the beneficial effect of overexpressing SERCA2a. *J. Biol. Chem.* **286,** 10163–10168 (2011).

15. Khatter, J. C., Sadri, P., Zhang, M. & Hoeschen, R. J. Myocardial Angiotensin II (Ang II) Receptors in Diabetic Rats. *Ann. N. Y. Acad. Sci.* **793,** 466–472 (1996).

16. Frustaci, A. *et al.* Myocardial Cell Death in Human Diabetes. *Circ. Res.* **87,** 1123–1132 (2000).

17. Davidoff, A. J., Davidson, M. B., Carmody, M. W., Davis, M. E. & Ren, J. Diabetic cardiomyocyte dysfunction and myocyte insulin resistance: role of glucose-induced PKC activity. *MolCell Biochem* **262,** 155–163 (2004).

18. Pan, Y. *et al.* Inhibition of JNK phosphorylation by a novel curcumin analog

prevents high glucose-induced inflammation and apoptosis in cardiomyocytes and the development of diabetic cardiomyopathy. *Diabetes* (2014).

19. Angin, Y. *et al.* CD36 inhibition prevents lipid accumulation and contractile dysfunction in rat cardiomyocytes. *Biochem.J* **448,** 43–53 (2012).

20. Boddu, N. J., Theus, S., Luo, S., Wei, J. Y. & Ranganathan, G. Is the lack of adiponectin associated with increased ER/SR stress and inflammation in the heart? *Adipocyte* **3,** 10–18 (2014).

21. Boudina, S. & Abel, E. D. Diabetic cardiomyopathy, causes and effects. *Rev. Endocr. Metab. Disord.* **11,** 31–39 (2010).

22. Bers, D. M. Ca transport during contraction and relaxation in mammalian ventricular muscle. *Basic Res. Cardiol.* **92,** 1–10 (1997).

23. Fabiato, A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am. J. Physiol.* **245,** C1–14 (1983).

24. Bers, D. M. Cardiac excitation–contraction coupling. *Nature* **415,** 198–205 (2002).

25. Lodish, H. *et al.* Myosin: The Actin Motor Protein. (2000).

26. Stammers, A. N. *et al.* The regulation of sarco(endo)plasmic reticulum calcium-ATPases (SERCA). *Can. J. Physiol. Pharmacol.* 1–12 (2015). doi:10.1139/cjpp-2014-0463

27. MacLennan, D. H. & Kranias, E. G. Phospholamban: a crucial regulator of cardiac contractility. *NatRevMolCell Biol* **4,** 566–577 (2003).

28. Tran, K., Smith, N. P., Loiselle, D. S. & Crampin, E. J. A thermodynamic model of the cardiac sarcoplasmic/endoplasmic Ca(2+) (SERCA) pump. *Biophys. J.* **96,** 2029–

2042 (2009).

29. Periasamy, M. & Kalyanasundaram, A. SERCA pump isoforms: Their role in calcium transport and disease. *Muscle Nerve* **35,** 430–442 (2007).

30. Toyoshima, C. & Inesi, G. Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. *Annu. Rev. Biochem.* **73,** 269–292 (2004).

31. Inesi, G., Lewis, D., Ma, H., Prasad, A. & Toyoshima, C. CONCERTED CONFORMATIONAL EFFECTS OF Ca 2+ AND ATP ARE REQUIRED FOR ACTIVATION OF SEQUENTIAL REACTIONS IN THE Ca2+ ATPase (SERCA) CATALYTIC CYCLE. *Biochemistry (Mosc.)* **45,** 13769–13778 (2006).

32. Rossi, A. E. & Dirksen, R. T. Sarcoplasmic reticulum: The dynamic calcium governor of muscle. *Muscle Nerve* **33,** 715–731 (2006).

33. Gunteski-Hamblin, A. M., Greeb, J. & Shull, G. E. A novel Ca2+ pump expressed in brain, kidney, and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum Ca-ATPase gene. Identification of cDNAs encoding Ca2+ and other cation-transporting ATPases using an oligonucleotide probe derived from the ATP-binding site. *J. Biol. Chem.* **263,** 15032–15040 (1988).

34. Vandecaetsbeek, I., Vangheluwe, P., Raeymaekers, L., Wuytack, F. & Vanoevelen, J. The Ca2+ Pumps of the Endoplasmic Reticulum and Golgi Apparatus. *Cold Spring Harb. Perspect. Biol.* **3,** a004184 (2011).

35. Bobe, R. *et al.* The rat platelet 97-kDa Ca2+ATPase isoform is the sarcoendoplasmic reticulum Ca2+ATPase 3 protein. *J. Biol. Chem.* **269,** 1417–1424 (1994).

36. Anger, M. *et al.* The sarco(endo)plasmic reticulum Ca2+-ATPase mRNA

isoform, SERCA 3, is expressed in endothelial and epithelial cells in various organs. *FEBS Lett.* **334,** 45–48 (1993).

37. Sumbilla, C. *et al.* Comparison of SERCA1 and SERCA2a expressed in COS-1 cells and cardiac myocytes. *Am. J. Physiol. - Heart Circ. Physiol.* **277,** H2381–H2391 (1999).

38. Lalli, M. J. *et al.* Sarcoplasmic Reticulum Ca2+ ATPase (SERCA) 1a Structurally Substitutes for SERCA2a in the Cardiac Sarcoplasmic Reticulum and Increases Cardiac Ca2+ Handling Capacity. *Circ. Res.* **89,** 160–167 (2001).

39. Pelled, D. *et al.* Inhibition of Calcium Uptake via the Sarco/Endoplasmic Reticulum Ca2+-ATPase in a Mouse Model of Sandhoff Disease and Prevention by Treatment with N-Butyldeoxynojirimycin. *J. Biol. Chem.* **278,** 29496–29501 (2003).

40. Naraynan, N. & Xu, A. Phosphorylation and regulation of the Ca2+-pumping ATPase in cardiac sarcoplasmic reticulum by calcium/calmodulin-dependent protein kinase. *Basic Res. Cardiol.* **92,** 25–35 (1997).

41. Roderick, H. L., Berridge, M. J. & Bootman, M. D. The Endoplasmic Reticulum: A Central Player in Cell Signalling and Protein Synthesis. in *Understanding Calcium Dynamics* (eds. Falcke, M. & Malchow, D.) 17–35 (Springer Berlin Heidelberg, 2003).

42. Periasamy, M., Bhupathy, P. & Babu, G. J. Regulation of sarcoplasmic reticulum Ca2+ ATPase pump expression and its relevance to cardiac muscle physiology and pathology. *Cardiovasc. Res.* **77,** 265–273 (2008).

43. Boudina, S. & Abel, E. D. Diabetic cardiomyopathy, causes and effects. *Rev. Endocr. Metab. Disord.* **11,** 31–39 (2010).

44. Suarez, J., Scott, B. & Dillmann, W. H. Conditional increase in SERCA2a protein

is able to reverse contractile dysfunction and abnormal calcium flux in established diabetic cardiomyopathy. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **295,** R1439–R1445 (2008).

45. Leszek, P. *et al.* Alteration of myocardial sarcoplasmic reticulum Ca2+-ATPase and Na+-Ca2+ exchanger expression in human left ventricular volume overload. *EurJ Heart Fail* **9,** 579–586 (2007).

46. del, M. *et al.* Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. *Circulation* **100,** 2308–2311 (1999).

47. Belke, D. D., Swanson, E. A. & Dillmann, W. H. Decreased sarcoplasmic reticulum activity and contractility in diabetic db/db mouse heart. *Diabetes* **53,** 3201–3208 (2004).

48. Zhao, S.-M., Wang, Y.-L., Guo, C.-Y., Chen, J.-L. & Wu, Y.-Q. Progressive decay of Ca2+ homeostasis in the development of diabetic cardiomyopathy. *Cardiovasc. Diabetol.* **13,** 75 (2014).

49. Epp, R. A. *et al.* Exercise training prevents the development of cardiac dysfunction in the low-dose streptozotocin diabetic rats fed a high-fat diet. *Can. J. Physiol. Pharmacol.* **91,** 80–89 (2012).

50. Jin, H. *et al.* QRS/T-Wave and Calcium Alternans in a Type I Diabetic Mouse Model for Spontaneous Post Myocardial Infarction Ventricular Tachycardia: A Mechanism for the Antiarrhythmic Effect of Statins. *Heart Rhythm* (2017). doi:10.1016/j.hrthm.2017.05.026

51. Cutler, M. J., Wan, X., Laurita, K. R., Hajjar, R. J. & Rosenbaum, D. S. Targeted SERCA2a gene expression identifies molecular mechanism and therapeutic target for

arrhythmogenic cardiac alternans. *Circ. Arrhythm. Electrophysiol.* **2,** 686–694 (2009).

52. Edwards, J. N. & Blatter, L. A. Cardiac alternans and intracellular calcium cycling. *Clin. Exp. Pharmacol. Physiol.* **41,** 524–532 (2014).

53. Roe, A., Frisk, M. & Louch, W. Targeting Cardiomyocyte Ca^{2+} Homeostasis in Heart Failure. *Curr. Pharm. Des.* **21,** 431–448 (2014).

54. Bers, D. M., Eisner, D. A. & Valdivia, H. H. Sarcoplasmic Reticulum Ca2+ and Heart Failure Roles of Diastolic Leak and Ca2+ Transport. *Circ. Res.* **93,** 487–490 (2003).

55. He, H. *et al.* Overexpression of the rat sarcoplasmic reticulum Ca2+ ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. *J. Clin. Invest.* **100,** 380–389 (1997).

56. Vetter, R. *et al.* Transgenic overexpression of the sarcoplasmic reticulum Ca2+ATPase improves reticular Ca2+ handling in normal and diabetic rat hearts. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **16,** 1657–1659 (2002).

57. Baker, D. L. *et al.* Targeted Overexpression of the Sarcoplasmic Reticulum Ca^{2+}-ATPase Increases Cardiac Contractility in Transgenic Mouse Hearts. *Circ. Res.* **83,** 1205–1214 (1998).

58. Schultz, J. E. J. *et al.* Accelerated onset of heart failure in mice during pressure overload with chronically decreased SERCA2 calcium pump activity. *Am. J. Physiol. - Heart Circ. Physiol.* **286,** H1146–H1153 (2004).

59. Münch, G. *et al.* SERCA2a activity correlates with the force-frequency relationship in human myocardium. *Am. J. Physiol. Heart Circ. Physiol.* **278,** H1924–1932 (2000).

60. Pharmacologic Management of Type 2 Diabetes: 2016 Interim Update. *Can. J. Diabetes* **40,** 193–195 (2016).

61. Jessup, M. *et al.* Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) A Phase 2 Trial of Intracoronary Gene Therapy of Sarcoplasmic Reticulum Ca2+-ATPase in Patients With Advanced Heart Failure. *Circulation* **124,** 304–313 (2011).

62. Greenberg, B. *et al.* Design of a Phase 2b Trial of Intracoronary Administration of AAV1/SERCA2a in Patients With Advanced Heart Failure: The CUPID 2 Trial (Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease Phase 2b). *JACC Heart Fail.* **2,** 84–92 (2014).

63. Greenberg, B. *et al.* Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (CUPID 2): a randomised, multinational, double-blind, placebo-controlled, phase 2b trial. *The Lancet* **387,** 1178–1186 (2016).

64. Mingozzi, F. *et al.* Overcoming Preexisting Humoral Immunity to AAV Using Capsid Decoys. *Sci. Transl. Med.* **5,** 194ra92–194ra92 (2013).

65. Watanabe, A. *et al.* Mitochondrial transcription factors TFAM and TFB2M regulate Serca2 gene transcription. *Cardiovasc. Res.* **90,** 57–67 (2011).

66. Choi, Y. S., Kim, S. & Pak, Y. K. Mitochondrial transcription factor A (mtTFA) and diabetes. *Diabetes Res. Clin. Pract.* **54,** S3–S9 (2001).

67. Ikeuchi, M. *et al.* Overexpression of Mitochondrial Transcription Factor A Ameliorates Mitochondrial Deficiencies and Cardiac Failure After Myocardial Infarction. *Circulation* **112,** 683–690 (2005).

68. Suarez, J. *et al.* Alterations in mitochondrial function and cytosolic calcium

induced by hyperglycemia are restored by mitochondrial transcription factor A in cardiomyocytes. *Am. J. Physiol. - Cell Physiol.* **295,** C1561–C1568 (2008).

69. Lumini-Oliveira, J. *et al.* Endurance training reverts heart mitochondrial dysfunction, permeability transition and apoptotic signaling in long-term severe hyperglycemia. *Mitochondrion* **11,** 54–63 (2011).

70. Sheng, T. & Yang, K. Adiponectin and its association with insulin resistance and type 2 diabetes. *J. Genet. Genomics Yi Chuan Xue Bao* **35,** 321–326 (2008).

71. Aleidi, S., Issa, A., Bustanji, H., Khalil, M. & Bustanji, Y. Adiponectin serum levels correlate with insulin resistance in type 2 diabetic patients. *Saudi Pharm. J. SPJ* **23,** 250–256 (2015).

72. Periasamy, M., Bhupathy, P. & Babu, G. J. Regulation of sarcoplasmic reticulum Ca2+ ATPase pump expression and its relevance to cardiac muscle physiology and pathology. *Cardiovasc. Res.* **77,** 265–273 (2008).

73. Zarain-Herzberg, A., Marques, J., Sukovich, D. & Periasamy, M. Thyroid hormone receptor modulates the expression of the rabbit cardiac sarco (endo) plasmic reticulum Ca(2+)-ATPase gene. *J. Biol. Chem.* **269,** 1460–1467 (1994).

74. Kimura, Y., Otsu, K., Nishida, K., Kuzuya, T. & Tada, M. Thyroid Hormone Enhances Ca2+ Pumping Activity of the Cardiac Sarcoplasmic Reticulum by Increasing Ca2+ ATPase and Decreasing Phospholamban Expression. *J. Mol. Cell. Cardiol.* **26,** 1145–1154 (1994).

75. Wang, C. The Relationship between Type 2 Diabetes Mellitus and Related Thyroid Diseases. *J. Diabetes Res.* **2013,** (2013).

76. Rai, S. *et al.* Thyroid Function In Type 2 Diabetes Mellitus and in Diabetic

Nephropathy. *J. Clin. Diagn. Res. JCDR* **7,** 1583–1585 (2013).

77. Duhamel, T. A. *et al.* Muscle metabolic, SR Ca(2+) -cycling responses to prolonged cycling, with and without glucose supplementation. *J ApplPhysiol* **103,** 1986–1998 (2007).

78. Bhupathy, P., Babu, G. J. & Periasamy, M. Sarcolipin and phospholamban as regulators of cardiac sarcoplasmic reticulum Ca2+ ATPase. *J. Mol. Cell. Cardiol.* **42,** 903–911 (2007).

79. Marks, A. R. Calcium and the heart: a question of life and death. *J. Clin. Invest.* **111,** 597–600 (2003).

80. Tsien, R. W. Cyclic AMP and contractile activity in heart. *Adv. Cyclic Nucleotide Res.* **8,** 363–420 (1977).

81. Drummond, G. I. & Severson, D. L. Cyclic nucleotides and cardiac function. *Circ. Res.* **44,** 145–153 (1979).

82. Koss, K. L., Ponniah, S., Jones, W. K., Grupp, I. L. & Kranias, E. G. Differential Phospholamban Gene Expression in Murine Cardiac Compartments Molecular and Physiological Analyses. *Circ. Res.* **77,** 342–353 (1995).

83. Kemi, O. J. *et al.* Aerobic interval training enhances cardiomyocyte contractility and Ca2+ cycling by phosphorylation of CaMKII and Thr-17 of phospholamban. *J. Mol. Cell. Cardiol.* **43,** 354–361 (2007).

84. Bupha-Intr, T., Laosiripisan, J. & Wattanapermpool, J. Moderate intensity of regular exercise improves cardiac SR Ca2+ uptake activity in ovariectomized rats. *J. Appl. Physiol.* **107,** 1105–1112 (2009).

85. Vasanji, Z., Dhalla, N. S. & Netticadan, T. Increased inhibition of SERCA2 by

phospholamban in the type I diabetic heart. *Mol. Cell. Biochem.* **261,** 245–249 (2004).

86. Vasanji, Z., Dhalla, N. S. & Netticadan, T. Increased inhibition of SERCA2 by phospholamban in the type I diabetic heart. *Mol. Cell. Biochem.* **261,** 245–249 (2004).

87. Hoshijima, M. *et al.* Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat. Med.* **8,** 864–871 (2002).

88. Huang, C. L. H. SERCA2a stimulation by istaroxime: a novel mechanism of action with translational implications. *Br. J. Pharmacol.* **170,** 486–488 (2013).

89. Ferrandi, M. *et al.* Istaroxime stimulates SERCA2a and accelerates calcium cycling in heart failure by relieving phospholamban inhibition. *Br. J. Pharmacol.* **169,** 1849–1861 (2013).

90. Micheletti, R. *et al.* Istaroxime, a stimulator of sarcoplasmic reticulum calcium adenosine triphosphatase isoform 2a activity, as a novel therapeutic approach to heart failure. *Am. J. Cardiol.* **99,** 24A–32A (2007).

91. George, M., Rajaram, M., Shanmugam, E. & VijayaKumar, T. M. Novel drug targets in clinical development for heart failure. *Eur. J. Clin. Pharmacol.* **70,** 765–774 (2014).

92. Asahi, M. *et al.* Sarcolipin regulates sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) by binding to transmembrane helices alone or in association with phospholamban. *Proc. Natl. Acad. Sci.* **100,** 5040–5045 (2003).

93. Sahoo, S. K. *et al.* The N-Terminus of Sarcolipin plays an important role in uncoupling Sarco-endoplasmic Reticulum Ca2+ ATPase (SERCA) ATP hydrolysis from Ca2+ transport. *J. Biol. Chem.* jbc.M115.636738 (2015). doi:10.1074/jbc.M115.636738

94. Tupling, A. R., Asahi, M. & MacLennan, D. H. Sarcolipin Overexpression in Rat Slow Twitch Muscle Inhibits Sarcoplasmic Reticulum Ca2+ Uptake and Impairs Contractile Function. *J. Biol. Chem.* **277,** 44740–44746 (2002).

95. Minamisawa, S. *et al.* Atrial Chamber-specific Expression of Sarcolipin Is Regulated during Development and Hypertrophic Remodeling. *J. Biol. Chem.* **278,** 9570–9575 (2003).

96. Zheng, J. *et al.* Increased Sarcolipin Expression and Adrenergic Drive in Humans With Preserved Left Ventricular Ejection Fraction and Chronic Isolated Mitral Regurgitation. *Circ. Heart Fail.* **7,** 194–202 (2014).

97. Babu, G. J. *et al.* Ablation of sarcolipin enhances sarcoplasmic reticulum calcium transport and atrial contractility. *Proc. Natl. Acad. Sci.* **104,** 17867–17872 (2007).

98. Babu, G. J. *et al.* Targeted Overexpression of Sarcolipin in the Mouse Heart Decreases Sarcoplasmic Reticulum Calcium Transport and Cardiac Contractility. *J. Biol. Chem.* **281,** 3972–3979 (2006).

99. Adachi, T. *et al.* S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat. Med.* **10,** 1200–1207 (2004).

100. Tang, W. H. *et al.* Cardiac contractile dysfunction during acute hyperglycemia due to impairment of SERCA by polyol pathway-mediated oxidative stress. *Am. J. Physiol. - Cell Physiol.* **299,** C643–C653 (2010).

101. Bidasee, K. R. *et al.* Diabetes Increases Formation of Advanced Glycation End Products on Sarco(endo)plasmic Reticulum Ca2+-ATPase. *Diabetes* **53,** 463–473 (2004).

102. Bennett, C. E., Johnsen, V. L., Shearer, J. & Belke, D. D. Exercise training mitigates aberrant cardiac protein O-GlcNAcylation in streptozotocin-induced diabetic

mice. *Life Sci.* **92,** 657–663 (2013).

103. Kho, C. *et al.* SUMO1-dependent modulation of SERCA2a in heart failure. *Nature* **477,** 601–605 (2011).

104. Tilemann, L. *et al.* SUMO-1 Gene Transfer Improves Cardiac Function in a Large-Animal Model of Heart Failure. *Sci. Transl. Med.* **5,** 211ra159–211ra159 (2013).

105. Foster, D. B. *et al.* The Cardiac Acetyl-Lysine Proteome. *PLoS ONE* **8,** (2013).

106. Al-Khalili, L., Krook, A. & Chibalin, A. V. Phosphorylation of the Na+,K+-ATPase in skeletal muscle: potential mechanism for changes in pump cell-surface abundance and activity. *Ann. N. Y. Acad. Sci.* **986,** 449–452 (2003).

107. Naraynan, N. & Xu, A. Phosphorylation and regulation of the Ca2+-pumping ATPase in cardiac sarcoplasmic reticulum by calcium/calmodulin-dependent protein kinase. *Basic Res. Cardiol.* **92,** 25–35 (1997).

108. Odermatt, A., Kurzydlowski, K. & MacLennan, D. H. The Vmax of the Ca2+-ATPase of Cardiac Sarcoplasmic Reticulum (SERCA2a) Is Not Altered by Ca2+/Calmodulin-dependent Phosphorylation or by Interaction with Phospholamban. *J. Biol. Chem.* **271,** 14206–14213 (1996).

109. Reddy, L. G., Jones, L. R., Pace, R. C. & Stokes, D. L. Purified, Reconstituted Cardiac Ca2+-ATPase Is Regulated by Phospholamban but Not by Direct Phosphorylation with Ca2+/Calmodulin-dependent Protein Kinase. *J. Biol. Chem.* **271,** 14964–14970 (1996).

110. Rodriguez, P., Jackson, W. A. & Colyer, J. Critical Evaluation of Cardiac Ca2+-ATPase Phosphorylation on Serine 38 Using a Phosphorylation Site-specific Antibody. *J. Biol. Chem.* **279,** 17111–17119 (2004).

111. Lancel, S. *et al.* Nitroxyl activates SERCA in cardiac myocytes via glutathiolation of cysteine 674. *Circ. Res.* **104,** 720–723 (2009).

112. Tong, X. *et al.* High glucose oxidizes SERCA cysteine-674 and prevents inhibition by nitric oxide of smooth muscle cell migration. *J. Mol. Cell. Cardiol.* **44,** 361–369 (2008).

113. Viner, R. I., Ferrington, D. A., Williams, T. D., Bigelow, D. J. & Schöneich, C. Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca2+-ATPase in skeletal muscle. *Biochem. J.* **340 (Pt 3),** 657–669 (1999).

114. Tang, W. H. *et al.* Cardiac contractile dysfunction during acute hyperglycemia due to impairment of SERCA by polyol pathway-mediated oxidative stress. *Am. J. Physiol. - Cell Physiol.* **299,** C643–C653 (2010).

115. Lokuta, A. J. *et al.* Increased Nitration of Sarcoplasmic Reticulum Ca^{2+}-ATPase in Human Heart Failure. *Circulation* **111,** 988–995 (2005).

116. Clark, R. J. *et al.* Diabetes and the Accompanying Hyperglycemia Impairs Cardiomyocyte Calcium Cycling through Increased Nuclear O-GlcNAcylation. *J. Biol. Chem.* **278,** 44230–44237 (2003).

117. Bidasee, K. R. *et al.* Diabetes Increases Formation of Advanced Glycation End Products on Sarco(endo)plasmic Reticulum Ca2+-ATPase. *Diabetes* **53,** 463–473 (2004).

118. Bennett, C. E., Johnsen, V. L., Shearer, J. & Belke, D. D. Exercise training mitigates aberrant cardiac protein O-GlcNAcylation in streptozotocin-induced diabetic mice. *Life Sci.* **92,** 657–663 (2013).

119. Yokoe, S. *et al.* Inhibition of phospholamban phosphorylation by O-

GlcNAcylation: implications for diabetic cardiomyopathy. *Glycobiology* **20,** 1217–1226 (2010).

120. Tong, X. *et al.* High glucose oxidizes SERCA cysteine-674 and prevents inhibition by nitric oxide of smooth muscle cell migration. *J. Mol. Cell. Cardiol.* **44,** 361–369 (2008).

121. Adachi, T. *et al.* S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat. Med.* **10,** 1200–1207 (2004).

122. Clark, R. J. *et al.* Diabetes and the Accompanying Hyperglycemia Impairs Cardiomyocyte Calcium Cycling through Increased Nuclear O-GlcNAcylation. *J. Biol. Chem.* **278,** 44230–44237 (2003).

123. Hu, Y. *et al.* Adenovirus-Mediated Overexpression of O-GlcNAcase Improves Contractile Function in the Diabetic Heart. *Circ. Res*. **96,** 1006–1013 (2005).

124. Rechem, C. V. *et al.* Differential Regulation of HIC1 Target Genes by CtBP and NuRD, via an Acetylation/SUMOylation Switch, in Quiescent versus Proliferating Cells. *Mol. Cell. Biol.* **30,** 4045–4059 (2010).

125. Kho, C. *et al.* Small-molecule activation of SERCA2a SUMOylation for the treatment of heart failure. *Nat. Commun.* **6,** (2015).

126. Lee, A., Oh, J. G., Gorski, P. A., Hajjar, R. J. & Kho, C. Post-translational Modifications in Heart Failure: Small Changes, Big Impact. *Heart Lung Circ.* **25,** 319–324 (2016).

127. Delcuve, G. P., Khan, D. H. & Davie, J. R. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. *Clin. Epigenetics* **4,** 5 (2012).

128. Glozak, M. A., Sengupta, N., Zhang, X. & Seto, E. Acetylation and deacetylation of non-histone proteins. *Gene* **363,** 15–23 (2005).

129. Hodawadekar, S. C. & Marmorstein, R. Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. *Oncogene* **26,** 5528–5540 (2007).

130. Choudhary, C. *et al.* Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions. *Science* **325,** 834–840 (2009).

131. Gu, W. & Roeder, R. G. Activation of p53 Sequence-Specific DNA Binding by Acetylation of the p53 C-Terminal Domain. *Cell* **90,** 595–606 (1997).

132. Jeninga, E. H., Schoonjans, K. & Auwerx, J. Reversible acetylation of PGC-1: Connecting energy sensors and effectors to guarantee metabolic flexibility. *Oncogene* **29,** (2010).

133. Fernandez-Marcos, P. J. & Auwerx, J. Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis1234. *Am. J. Clin. Nutr.* **93,** 884S–890S (2011).

134. Philp, A., Rowland, T., Perez-Schindler, J. & Schenk, S. Understanding the acetylome: translating targeted proteomics into meaningful physiology. *Am. J. Physiol. - Cell Physiol.* **307,** C763–C773 (2014).

135. Susser, S. Examining the effects of SERCA2a acetylation in the heart. (University of Manitoba, 2016).

136. Spange, S., Wagner, T., Heinzel, T. & Krämer, O. H. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int. J. Biochem. Cell Biol.* **41,** 185–198 (2009).

137. North, B. J. & Verdin, E. Sirtuins: Sir2-related NAD-dependent protein

deacetylases. *Genome Biol.* **5,** 224 (2004).

138. Dokmanovic, M., Clarke, C. & Marks, P. A. Histone Deacetylase Inhibitors: Overview and Perspectives. *Mol. Cancer Res.* **5,** 981–989 (2007).

139. Guarente, L. Sirtuins, Aging, and Metabolism. *Cold Spring Harb. Symp. Quant. Biol.* **76,** 81–90 (2011).

140. Schölz, C. *et al.* Acetylation site specificities of lysine deacetylase inhibitors in human cells. *Nat. Biotechnol.* **33,** 415–423 (2015).

141. Furumai, R. *et al.* FK228 (Depsipeptide) as a Natural Prodrug That Inhibits Class I Histone Deacetylases. *Cancer Res.* **62,** 4916–4921 (2002).

142. Nebbioso, A. *et al.* Selective class II HDAC inhibitors impair myogenesis by modulating the stability and activity of HDAC–MEF2 complexes. *EMBO Rep.* **10,** 776–782 (2009).

143. Sack, M. N. & Finkel, T. Mitochondrial Metabolism, Sirtuins, and Aging. *Cold Spring Harb. Perspect. Biol.* **4,** a013102 (2012).

144. Bolden, J. E., Peart, M. J. & Johnstone, R. W. Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* **5,** 769–784 (2006).

145. Rotili, D. *et al.* Non-cancer uses of histone deacetylase inhibitors: effects on infectious diseases and beta-hemoglobinopathies. *Curr. Top. Med. Chem.* **9,** 272–291 (2009).

146. Cao, D. J. *et al.* Histone deacetylase (HDAC) inhibitors attenuate cardiac hypertrophy by suppressing autophagy. *Proc. Natl. Acad. Sci.* **108,** 4123–4128 (2011).

147. Nural-Guvener, H. F. *et al.* HDAC class I inhibitor, Mocetinostat, reverses cardiac fibrosis in heart failure and diminishes CD90+ cardiac myofibroblast activation.

Fibrogenesis Tissue Repair **7,** 10 (2014).

148. Haigis, M. C. & Sinclair, D. A. Mammalian Sirtuins: Biological Insights and Disease Relevance. *Annu. Rev. Pathol.* **5,** 253–295 (2010).

149. Zhang, H. *et al.* NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science* **352,** 1436–1443 (2016).

150. Guarente, L. The resurgence of NAD+. *Science* **352,** 1396–1397 (2016).

151. Tanno, M., Kuno, A., Horio, Y. & Miura, T. Emerging beneficial roles of sirtuins in heart failure. *Basic Res. Cardiol.* **107,** (2012).

152. Sundaresan, N. R., Samant, S. A., Pillai, V. B., Rajamohan, S. B. & Gupta, M. P. SIRT3 Is a Stress-Responsive Deacetylase in Cardiomyocytes That Protects Cells from Stress-Mediated Cell Death by Deacetylation of Ku70. *Mol. Cell. Biol.* **28,** 6384–6401 (2008).

153. Sundaresan, N. R. *et al.* The sirtuin SIRT6 blocks IGF-Akt signaling and development of cardiac hypertrophy by targeting c-Jun. *Nat. Med.* **18,** 1643–1650 (2012).

154. Yu, W., Zhou, H.-F., Lin, R.-B., Fu, Y.-C. & Wang, W. Short-term calorie restriction activates SIRT1-4 and -7 in cardiomyocytes in vivo and in vitro. *Mol. Med. Rep.* **9,** 1218–1224 (2014).

155. Tanno, M., Sakamoto, J., Miura, T., Shimamoto, K. & Horio, Y. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. *J BiolChem* **282,** 6823–6832 (2007).

156. Alcendor, R. R. *et al.* Sirt1 Regulates Aging and Resistance to Oxidative Stress in the Heart. *Circ. Res.* **100,** 1512–1521 (2007).

157. Xu, F. *et al.* Lack of SIRT1 (Mammalian Sirtuin 1) Activity Leads to Liver

Steatosis in the SIRT1+/− Mice: A Role of Lipid Mobilization and Inflammation. *Endocrinology* **151,** 2504–2514 (2010).

158. Bordone, L. *et al.* SIRT1 transgenic mice show phenotypes resembling calorie restriction. *Aging Cell* **6,** 759–767 (2007).

159. Suwa, M., Nakano, H., Radak, Z. & Kumagai, S. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor γ coactivator-1α protein expressions in rat skeletal muscle. *Metabolism* **57,** 986–998 (2008).

160. Ferrara, N. *et al.* Exercise training promotes SIRT1 activity in aged rats. *Rejuvenation Res.* **11,** 139–150 (2008).

161. Sulaiman, M. *et al.* Resveratrol, an activator of SIRT1, upregulates sarcoplasmic calcium ATPase and improves cardiac function in diabetic cardiomyopathy. *Am. J. Physiol. - Heart Circ. Physiol.* **298,** H833–H843 (2010).

162. Hernández, J. S., Barreto-Torres, G., Kuznetsov, A. V., Khuchua, Z. & Javadov, S. Crosstalk between AMPK activation and angiotensin II-induced hypertrophy in cardiomyocytes: the role of mitochondria. *J. Cell. Mol. Med.* **18,** 709–720 (2014).

163. Hirschey, M. D. *et al.* SIRT3 Deficiency and Mitochondrial Protein Hyperacetylation Accelerate the Development of the Metabolic Syndrome. *Mol. Cell* **44,** 177–190 (2011).

164. Sundaresan, N. R. *et al.* Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J. Clin. Invest.* **119,** 2758–2771 (2009).

165. Parodi-Rullán, R. M., Chapa-Dubocq, X., Rullán, P. J., Jang, S. & Javadov, S. High Sensitivity of SIRT3 Deficient Hearts to Ischemia-Reperfusion Is Associated with

Mitochondrial Abnormalities. *Front. Pharmacol.* **8,** (2017).

166. Turkmen, K., Karagoz, A. & Kucuk, A. Sirtuins as novel players in the pathogenesis of diabetes mellitus. *World J. Diabetes* **5,** 894–900 (2014).

167. Vargas-Ortiz, K. *et al.* Aerobic Training Increases Expression Levels of SIRT3 and PGC-1α in Skeletal Muscle of Overweight Adolescents Without Change in Caloric Intake. *Pediatr. Exerc. Sci.* (2015). doi:10.1123/pes.2014-0112

168. Huynh, F. K., Hershberger, K. A. & Hirschey, M. D. Targeting sirtuins for the treatment of diabetes. *Diabetes Manag. Lond. Engl.* **3,** 245–257 (2013).

169. Berndsen, C. E. & Denu, J. M. Catalysis and substrate selection by histone/protein lysine acetyltransferases. *Curr. Opin. Struct. Biol.* **18,** 682–689 (2008).

170. Roth, S. Y., Denu, J. M. & Allis, C. D. Histone acetyltransferases. *Annu. Rev. Biochem.* **70,** 81–120 (2001).

171. Coste, A. *et al.* The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1α. *Proc. Natl. Acad. Sci.* **105,** 17187–17192 (2008).

172. Moreno, M. *et al.* PPARs: Nuclear Receptors Controlled by, and Controlling, Nutrient Handling through Nuclear and Cytosolic Signaling. *PPAR Res.* **2010,** e435689 (2010).

173. Moreno, M. *et al.* PPARs: Nuclear Receptors Controlled by, and Controlling, Nutrient Handling through Nuclear and Cytosolic Signaling. *PPAR Res.* **2010,** e435689 (2010).

174. Lerin, C. *et al.* GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1α. *Cell Metab.* **3,** 429–438 (2006).

175. Kelly, T. J., Lerin, C., Haas, W., Gygi, S. P. & Puigserver, P. GCN5-mediated Transcriptional Control of the Metabolic Coactivator PGC-1β through Lysine Acetylation. *J. Biol. Chem.* **284,** 19945 (2009).

176. Kong, X. *et al.* Sirtuin 3, a New Target of PGC-1α, Plays an Important Role in the Suppression of ROS and Mitochondrial Biogenesis. *PLOS ONE* **5,** e11707 (2010).

177. Pillai, V. B. *et al.* Exogenous NAD Blocks Cardiac Hypertrophic Response via Activation of the SIRT3-LKB1-AMP-activated Kinase Pathway. *J. Biol. Chem.* **285,** 3133–3144 (2010).

178. Ruderman, N. B. *et al.* AMPK and SIRT1: a long-standing partnership? *Am. J. Physiol. - Endocrinol. Metab.* **298,** E751–E760 (2010).

179. Brandauer, J. *et al.* AMP-activated protein kinase regulates nicotinamide phosphoribosyl transferase expression in skeletal muscle. *J. Physiol.* **591,** 5207–5220 (2013).

180. Philp, A. *et al.* Sirtuin 1 (SIRT1) Deacetylase Activity Is Not Required for Mitochondrial Biogenesis or Peroxisome Proliferator-activated Receptor-γ Coactivator-1α (PGC-1α) Deacetylation following Endurance Exercise. *J. Biol. Chem.* **286,** 30561–30570 (2011).

181. Black, J. C., Mosley, A., Kitada, T., Washburn, M. & Carey, M. The SIRT2 Deacetylase Regulates Autoacetylation of p300. *Mol. Cell* **32,** 449–455 (2008).

182. Shin, J., Zhang, D. & Chen, D. Reversible Acetylation of Metabolic Enzymes Celebration: SIRT2 and p300 Join the Party. *Mol. Cell* **43,** 3–5 (2011).

183. Han, Y. *et al.* Acetylation of Sirt2 by p300 attenuates its deacetylase activity. *Biochem. Biophys. Res. Commun.* **375,** 576–580 (2008).

184. Nemoto, S., Fergusson, M. M. & Finkel, T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. *J. Biol. Chem.* **280,** 16456–16460 (2005).

185. Wang, J. & Chen, J. SIRT1 Regulates Autoacetylation and Histone Acetyltransferase Activity of TIP60. *J. Biol. Chem.* **285,** 11458–11464 (2010).

186. Yamagata, K. & Kitabayashi, I. Sirt1 physically interacts with Tip60 and negatively regulates Tip60-mediated acetylation of H2AX. *Biochem. Biophys. Res. Commun.* **390,** 1355–1360 (2009).

187. Peng, L. *et al.* SIRT1 Negatively Regulates the Activities, Functions, and Protein Levels of hMOF and TIP60. *Mol. Cell. Biol.* **32,** 2823–2836 (2012).

188. Tang, Y., Luo, J., Zhang, W. & Gu, W. Tip60-Dependent Acetylation of p53 Modulates the Decision between Cell-Cycle Arrest and Apoptosis. *Mol. Cell* **24,** 827–839 (2006).

189. Cui, L. *et al.* Histone Acetyltransferase Inhibitor Anacardic Acid Causes Changes in Global Gene Expression during In Vitro Plasmodium falciparum Development. *Eukaryot. Cell* **7,** 1200–1210 (2008).

190. Chandregowda, V., Kush, A. & Reddy, G. C. Synthesis of benzamide derivatives of anacardic acid and their cytotoxic activity. *Eur. J. Med. Chem.* **44,** 2711–2719 (2009).

191. Ghizzoni, M. *et al.* 6-alkylsalicylates are selective Tip60 inhibitors and target the acetyl-CoA binding site. *Eur. J. Med. Chem.* **47,** 337–344 (2012).

192. Bowers, E. M. *et al.* Virtual Ligand Screening of the p300/CBP Histone Acetyltransferase: Identification of a Selective Small Molecule Inhibitor. *Chem. Biol.* **17,** 471–482 (2010).

193. Chimenti, F. *et al.* A novel histone acetyltransferase inhibitor modulating Gcn5 network: cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl)hydrazone. *J. Med. Chem.* **52,** 530–536 (2009).

194. Wu, Y. *et al.* Loss of GCN5 leads to increased neuronal apoptosis by upregulating E2F1- and Egr-1-dependent BH3-only protein Bim. *Cell Death Dis.* **8,** e2570 (2017).

195. Louch, W. E., Sheehan, K. A. & Wolska, B. M. Methods in Cardiomyocyte Isolation, Culture, and Gene Transfer. *J. Mol. Cell. Cardiol.* **51,** 288–298 (2011).

196. Cardi, D. *et al.* Purified E255L Mutant SERCA1a and Purified PfATP6 Are Sensitive to SERCA-type Inhibitors but Insensitive to Artemisinins. *J. Biol. Chem.* **285,** 26406–26416 (2010).

197. Seidler, N. W., Jona, I., Vegh, M. & Martonosi, A. Cyclopiazonic acid is a specific inhibitor of the Ca2+-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **264,** 17816–17823 (1989).

198. Gang, H., Shaw, J., Dhingra, R., Davie, J. R. & Kirshenbaum, L. A. Epigenetic regulation of canonical TNFα pathway by HDAC1 determines survival of cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol.* **304,** H1662–1669 (2013).

199. van den Bosch, T. *et al.* A 6-alkylsalicylate histone acetyltransferase inhibitor inhibits histone acetylation and pro-inflammatory gene expression in murine precision-cut lung slices. *Pulm. Pharmacol. Ther.* **44,** 88–95 (2017).

200. Kranias, E. G. & Hajjar, R. J. Modulation of Cardiac Contractility by the Phopholamban/SERCA2a Regulatome. *Circ. Res.* **110,** 1646–1660 (2012).

201. Maruyama, K. & MacLennan, D. H. Mutation of aspartic acid-351, lysine-352, and lysine-515 alters the Ca2+ transport activity of the Ca2+-ATPase expressed in COS-1

cells. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3314–3318 (1988).

202. Kho, C. *et al.* SUMO1-dependent modulation of SERCA2a in heart failure. *Nature* **477,** 601–605 (2011).

203. Akin, B. L., Hurley, T. D., Chen, Z. & Jones, L. R. The Structural Basis for Phospholamban Inhibition of the Calcium Pump in Sarcoplasmic Reticulum. *J. Biol. Chem.* **288,** 30181–30191 (2013).

204. Gustavsson, M. *et al.* Allosteric regulation of SERCA by phosphorylation-mediated conformational shift of phospholamban. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 17338–17343 (2013).

205. Sulaiman, M. *et al.* Resveratrol, an activator of SIRT1, upregulates sarcoplasmic calcium ATPase and improves cardiac function in diabetic cardiomyopathy. *Am. J. Physiol. - Heart Circ. Physiol.* **298,** H833–H843 (2010).

206. Hershberger, K. A., Martin, A. S. & Hirschey, M. D. Role of NAD(+) and mitochondrial sirtuins in cardiac and renal diseases. *Nat. Rev. Nephrol.* **13,** 213–225 (2017).

207. Schwer, B., Bunkenborg, J., Verdin, R. O., Andersen, J. S. & Verdin, E. Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc. Natl. Acad. Sci.* **103,** 10224–10229 (2006).

208. Huang, C. L. H. SERCA2a stimulation by istaroxime: a novel mechanism of action with translational implications. *Br. J. Pharmacol.* **170,** 486–488 (2013).

209. Tanno, M., Sakamoto, J., Miura, T., Shimamoto, K. & Horio, Y. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. *J BiolChem* **282,** 6823–6832 (2007).

210. Yu, W., Zhou, H.-F., Lin, R.-B., Fu, Y.-C. & Wang, W. Short-term calorie restriction activates SIRT1-4 and -7 in cardiomyocytes in vivo and in vitro. *Mol. Med. Rep.* **9,** 1218–1224 (2014).

211. Sundaresan, N. R., Samant, S. A., Pillai, V. B., Rajamohan, S. B. & Gupta, M. P. SIRT3 Is a Stress-Responsive Deacetylase in Cardiomyocytes That Protects Cells from Stress-Mediated Cell Death by Deacetylation of Ku70. *Mol. Cell. Biol.* **28,** 6384–6401 (2008).

212. Morissette, M. P. *et al.* Differential regulation of the fiber type specific gene expression of the sarcoplasmic reticulum calcium-ATPase (SERCA) isoforms induced by exercise training. *J ApplPhysiol 1985* (2014).

213. Palacios, O. M. *et al.* Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. *Aging AlbanyNY* **1,** 771–783 (2009).

214. Dokmanovic, M., Clarke, C. & Marks, P. A. Histone Deacetylase Inhibitors: Overview and Perspectives. *Mol. Cancer Res.* **5,** 981–989 (2007).

215. Ooi, J. Y. Y. *et al.* HDAC inhibition attenuates cardiac hypertrophy by acetylation and deacetylation of target genes. *Epigenetics* **10,** 418–430 (2015).