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Hormonal Regulation Of Glycine Decarboxylase And Its Metabolic Outcomes

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HORMONAL REGULATION OF GLYCINE DECARBOXYLASE AND ITS METABOLIC OUTCOMES

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DISSERTATION

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DEDICATION

I dedicate this thesis to

my family for their support, encouragement, and unconditional love.

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PREFACE

Numerous studies have consistently demonstrated a significant reduction in the circulating levels of the amino acid, glycine in diabetes, and prediabetic states. Even though glycine is a chemically simple, non-essential amino acid, it plays an essential role in multiple critical metabolic pathways such as the biosynthesis of glutathione, heme, creatine, purines, and other metabolites. Together, these observations led us to ask the basic question underlying this thesis: do changes in glycine metabolism play a role in the development of type 2 diabetes and related metabolic diseases.

The numerous important metabolic roles for glycine suggest that its levels must be maintained within a specific concentration range to ensure availability for these functions. Glycine levels are maintained by balancing dietary intake, biosynthesis from metabolic intermediates, and degradation. Information from naturally occurring mutations in the glycine degradation system have demonstrated that the level of glycine in circulation is controlled primarily by the rate of its degradation. My thesis work focuses on the rate-limiting enzyme of this glycine cleavage system- glycine decarboxylase (GLDC). Modulating GLDC levels *in vitro* and *in vivo* are known to alter circulating glycine levels. The general hypothesis that forms the basis for my thesis work is that there is a functional relationship between altered GLDC levels and the development of diabetes.

As described in this dissertation, we demonstrated that GLDC expression is upregulated in rodent models of metabolic stress; diabetes, obesity, and fasting (described in chapter 2), and we went on to show that GLDC expression is regulated by the metabolic hormones, glucagon, and insulin (chapter 3).

Finally, we addressed potential links between GLDC expression and parameters related to metabolic health by demonstrating that changes in GLDC expression are strongly linked to glutathione production and cellular oxidative stress levels (chapter 4). Each of these chapters, which represent the three specific aims of my thesis project, contain their own introduction, methods, results, and discussion sections. These 'results' chapters are preceded by a background chapter providing an overview of glycine metabolism and followed by a conclusion chapter describing the general significance of this work and future directions.

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LIST OF ABBREVIATIONS

ad lib	ad libitum
AMT	Aminomethyl transferase
ATF1	Activating transcription factor 1
BCA	bicinchoninic
CREB1	cAMP Responsive Element Binding Protein 1
db/db	Mice homozygous for diabetes Leprdb mutations
DLD	dihydrolipoamide dehydrogenase
DMEM	Dulbecco's modified eagle medium
dT2D	type 2 diabetic
EPIC	European Prospective Investigation into Cancer and Nutrition
FASN	fatty acid synthase
FBS	fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCSH	Glycine cleavage system H-protein
GEO	Gene Expression Omnibus
GLDC	Glycine decarboxylase
GSH	Glutathione reduced
GSSG	Glutathione oxidized
HRP	Horseradish peroxidase
IGT	Impaired glucose tolerance
meTHF	methylene tetrahydrofolate
NADH	Reduced form of Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NAFLD	Non-alcoholic fatty liver disease
NCBI	National Center for Biotechnology Information
NGT	Normal glucose tolerance
NKH	non-ketotic hyperglycinemia
NMDA	N-methyl-D-aspartate
NSCLC	non-small cell lung carcinoma
ob/ob	Mice homozygous for obese Leprob mutations
PCK1	phosphoenolpyruvate carboxykinase
PKA	protein kinase a
PPIA	peptidylprolyl isomerase A
PPIB	peptidylprolyl isomerase B
qRT-PCR	quantitative real time polymerase chain reaction
ROS	reactive oxygen species
S.D.	Standard deviation
sgRNA	single guide RNA
SHMT1	serine hydroxymethyltransferase 1
SHMT2	serine hydroxymethyltransferase 2
SREBP1c	Sterol regulatory element binding protein 1c
TBP	Tata binding protein
TDH	threonine dehydrogenase
THF	tetrahydrofolate

CHAPTER 1 – BACKGROUND

The work described in this thesis is centered on issues related to the amino acid glycine, and its metabolism. The observations that formed the impetus for this project are that glycine levels are consistently reduced in states of metabolic stress such as diabetes and obesity. This led to our overall hypothesis that there are unrecognized functional links between glycine metabolism and the maintenance of metabolic homeostasis. To more fully appreciate how the experiments described in this dissertation were designed, and the data interpreted, I am providing this overview of glycine, its physiological functions and metabolic fates.

1.1 Glycine metabolism is altered in diabetes and related metabolic disorders

Insulin resistance, defined as a reduced sensitivity or responsiveness to insulin, is a crucial step in the development of the metabolic syndrome (obesity, hyperglycemia, dyslipidemia, hypertension, and cardiovascular disease) and type 2 diabetes (1, 2). The diabetic state is accompanied by alterations in glucose, lipid, and even protein metabolic pathways. It is now well-established that the levels of certain amino acids are consistently altered in insulin resistance and diabetes (3-5) (Figure 1.1). Altered metabolite profiles can accompany changes in metabolic pathways in the disease state. A consistent change in such metabolites can be utilized, in the form of ‘biomarkers’, as predictors of a metabolic dysregulation Towards this goal, large-scale metabolomic studies were undertaken in the last 15 years with the aim to identify potential metabolites to predict type 2 diabetes risk.

	Metabolic pathways	Diseases	Association
Glycine	Alanine	Insulin resistance	-
	Glutathione	Obesity	-
	Glycine and serine	Diabetes	-
	Bile-acid biosynthesis		
	Methionine		
Isoleucine	BCAA catabolism	Insulin resistance	+
		Obesity	+
		Diabetes	+
Leucine	BCAA catabolism	Insulin resistance	+
		Obesity	+
		Diabetes	+
Valine	BCAA catabolism	Insulin resistance	+
		Obesity	+
		Diabetes	+

Figure 1.1: Amino acids and amino acid pathways associated with insulin resistance, type 2 diabetes, and obesity.
Adapted from (5)

Typically, a diabetes metabolomic study design consists of measurement of statistically significant differences in metabolite concentration from a blood sample taken at a single time point from healthy individuals and diabetic patients. But for the identification of biomarkers for type 2 diabetes risk, cross-sectional and longitudinal study designs were utilized to guarantee a more heterogeneous group of participants over a longer study duration. These studies identified branched-chain amino acids (valine, leucine, and isoleucine) as effective markers of risk of type 2 diabetes. They are positively correlated with obesity and insulin levels. The only amino acid that was found to be negatively correlated with type 2 diabetes risk, obesity, and insulin resistance in these studies was glycine (6-9).

A longitudinal population-based study was conducted by Wang-Sattler et al. (2012), where the group used 4297 serum samples taken over a span of 7 years and analyzed 140 metabolites. This study identified reduced glycine levels as a candidate biomarker for impaired glucose tolerance and type 2 diabetic state (Figure 1.2) (10). Another study by Floegel et al. (2013) utilized targeted metabolomics on a randomly drawn sample set of 2282 controls and 800 type 2 diabetic cases from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam. This study used an average follow-up time of 7 years. Reduced glycine levels were identified as significant predictors of type 2 diabetes development (11). Another metabolite profiling using a longitudinal community-based Framingham Heart Study was carried out by Cheng et al. (2012). This cohort had n=1015 type 2 diabetes and cardiovascular disease-free individuals who had high-throughput, unbiased metabolite profiling performed as part of the study to identify metabolic disease risk.

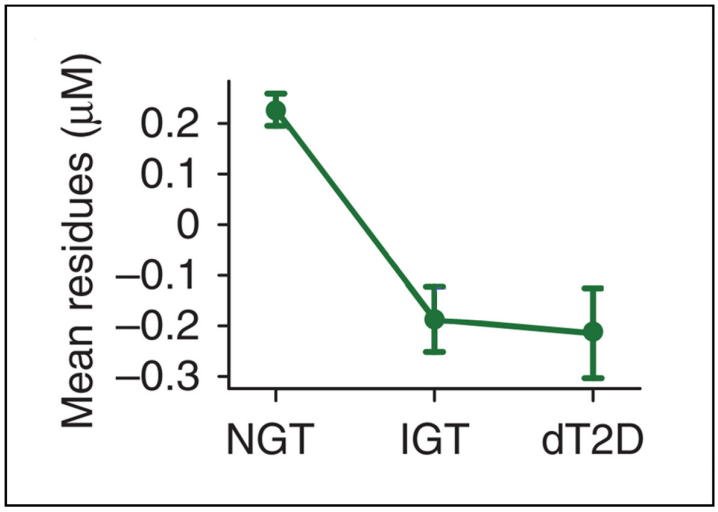

Figure 1.2: Glycine levels during progression to type 2 diabetes.
The mean residues of the glycine concentration (μM) with standard errors for the normal glucose tolerant (NGT), impaired glucose tolerant (IGT) and type 2 diabetic (dT2D) groups Adapted from (10).

Glycine was significantly and inversely associated with the multiple components of metabolic disease – body mass index, waist circumference, insulin, homeostasis model assessment of insulin resistance, systolic and diastolic blood pressure, and triglycerides (12). A community-based multiethnic (European American, Hispanic, and African American) cohort with an n=196 from the Insulin Resistance Atherosclerosis Study was utilized by Palmer et al., (2015). Amongst the metabolites profiled, decreased circulating glycine was consistently observed in individuals that converted to an insulin resistant and type 2 diabetic phenotype (13). Non-alcoholic fatty liver disease (NAFLD) is strongly associated with metabolic syndromes- insulin resistance, obesity, and type 2 diabetes. Hepatic steatosis is a characteristic feature of NAFLD. In a study with n=86 individuals with varying degrees of hepatic steatosis, fasting circulating glycine levels show a significantly negative correlation with hepatic steatosis (13).

In summary, these findings, together with multiple studies not described here, clearly demonstrate that circulating glycine levels are altered in type 2 diabetes and multiple metabolic syndrome traits. One of the questions we seek to answer is whether these findings are incidental to the development of diabetes or if they play a direct functional role in the development of the disease state.

A potential causative role for reduced glycine levels in the development of metabolic disease comes from studies showing that interventions that either delay or reverse the onset of type 2 diabetes are associated with an increase in circulating glycine levels. For instance, individuals who have undergone bariatric surgery, as a treatment option for obesity, which in many cases can reverse the onset of type 2 diabetes, show a significant increase in plasma glycine levels (14).

Another example is of exercise intervention in obese individuals, which increased circulating glycine levels as compared to the obese subjects who did not train (15). Even more suggestive are studies showing that glycine supplementation improves insulin response and glucose tolerance. Glycine administration has been shown to enhance glucose tolerance in healthy subjects and reduce glycosylated hemoglobin in patients with type 2 diabetes (reviewed in (16)). Oral glycine supplementation has been shown to increase glucagon concentration and dramatically reduce the glucose area response (17). Glycine is one of the precursors of the antioxidant glutathione biosynthesis (as explained in the later sections). In diabetic patients and in aged individuals, a deficiency of the glutathione precursor, glycine, could explain the oxidative stress and damage observed in these individuals.

Supplementing the diets of elderly individuals with glycine for 14 days restored glutathione synthesis and pools to levels comparable to younger individuals and thereby reducing oxidative damage (18). In another study, 12 diabetic and 12 non-diabetic subjects were infused with [$^2H_2$]-glycine to measure glutathione synthesis, intracellular concentrations and markers of oxidative stress. Dietary supplementation with glycine for 2 weeks enhanced the antioxidant defenses of the cells and restored the synthesis of glutathione to a large extent (19).

Clearly, glycine levels are a reliable indicator of metabolic health. In the next section, we will look at some of the metabolic roles and fates of glycine.

1.2 Physiological roles of glycine

Glycine is the simplest amino acid with hydrogen in place of a side chain common to all other amino acids. It is considered a non-essential amino acid as it is synthesized endogenously from various sources. But some studies show that the metabolic demand for glycine is greater than the synthesis rate. Although mild insufficiency of glycine is not threatening for life, a chronic shortage may result in suboptimal growth, impaired immune responses, and other adverse effects on health and nutrient metabolism. The following is an overview of some of the known physiological functions of glycine.

1.2.1 **Glycine participates in protein biosynthesis**

In growing animals, 80% of glycine is used for protein synthesis. Glycine has a unique conformational advantage in enzymatic structure and function. Glycine residues are not restricted in their conformational freedom as compared to other amino acids with bulky side chains, and therefore, can provide a wide degree of flexibility for adjacent residues. This is especially useful for active site regions within enzymes where glycine residues provide the flexibility necessary for active site conformational changes (20). Collagen, the most abundant protein in the human body, is composed of 35% glycine (21). Glycine plays a crucial role in stabilizing the triple helical structure of collagen. For the formation of healthy collagen, every third amino acid in its superhelical structure is glycine to allow close proximity of the three chains (22).

1.2.2 **Glycine is required for the first step in heme biosynthesis**

The first and rate-limiting step of heme biosynthesis takes place in the mitochondria where glycine condenses with succinyl-coA from the Kreb's cycle forming delta-aminolevulinic acid (23). Eight glycine molecules are needed for the synthesis of one heme molecule. Heme is a component of various proteins in the electron transfer chain ensuring mitochondrial protein stability and function. A decrease in mitochondrial heme content could lead to mitochondrial dysfunction and reduced respiratory rates (24). Heme is a physiological ligand for Rev-erbα, which regulates several genes in the circadian pathway and lipid metabolism. Rev-erbα can sense and utilize heme to integrate circadian and metabolic pathways (25). Reticulocytes import glycine through the glycine transporter 1 for heme biosynthesis. An insufficiency of glycine supply to the erythroid cells causes a decline in heme production. Disruption of the glycine transporter in mice caused a lethal phenotype by the development of microcytic anemia (26). The fact that interruption in glycine supply to the erythroid cells is lethal supports that glycine uptake is critical and that endogenous production of glycine is insufficient to keep up with the metabolic demand for glycine (26).

1.2.3 **Glycine is required for glutathione biosynthesis**

The antioxidant glutathione is a tripeptide that consists of glutamate, cysteine, and glycine. It is present in all mammalian tissues, but its levels are especially higher in the liver (27). The liver plays a critical role in interorgan glutathione homeostasis (28). Glutathione exists in either the predominant reduced (GSH) form present in millimolar quantities in the cell or the oxidized (GSSG) form which comprises 1% of the glutathione pool (29).

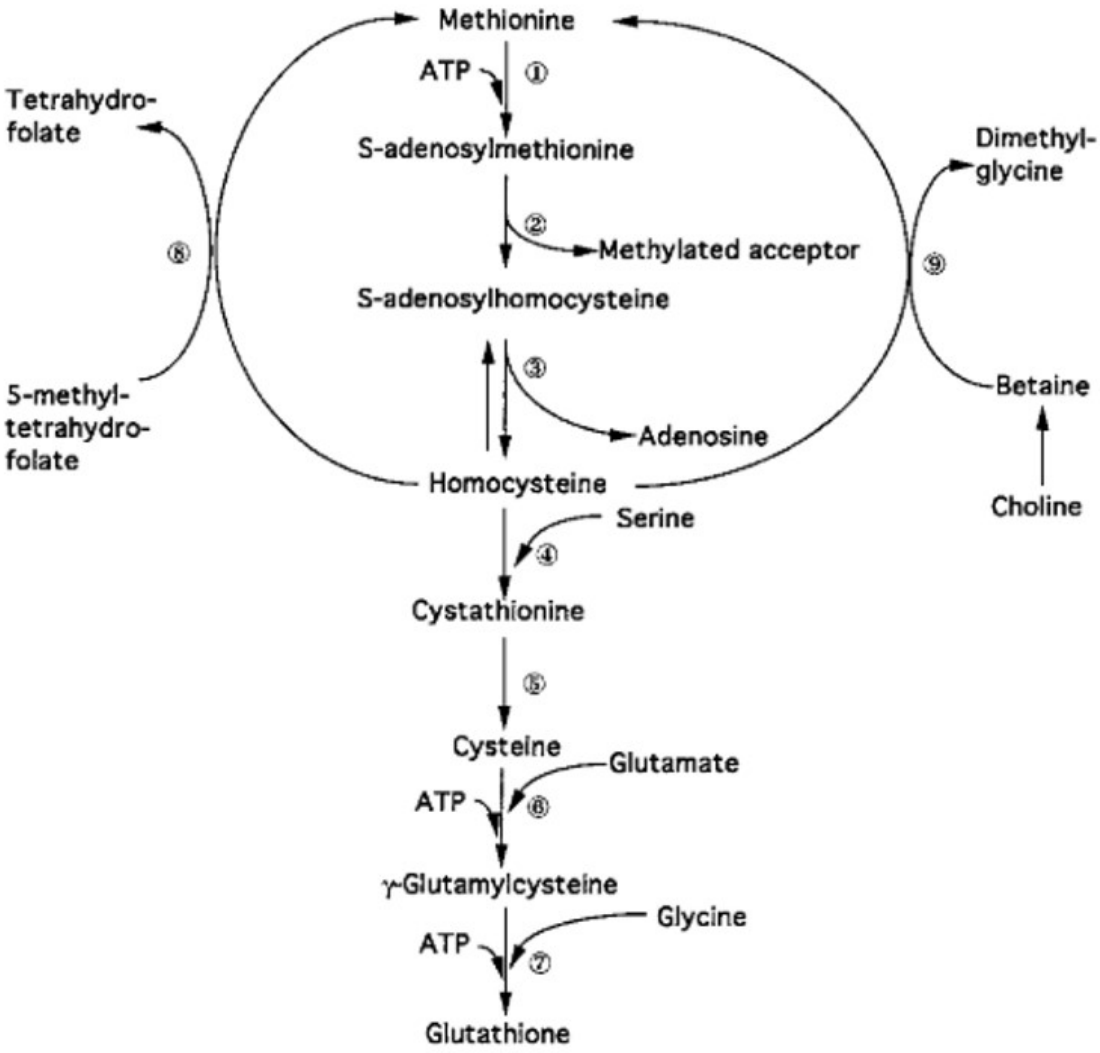

Figure 1.3: Methionine and transsulfuration pathway
Methionine is converted to cysteine and eventually into glutathione by the methionine-transsulfuration pathway. The enzyme depicted (1), methionine adenosyltransferase; (2), transmethylation reactions; (3), S-adenosylhomocysteine hydrolase; (4), cystathione synthase;(5), cystathionase; (6), glutamylcysteine synthetase; (7), GSH synthetase; (8), methionine synthase; (9), betaine-homocysteine methyltransferase (30).

Within the cell, about 85-90% of glutathione is compartmentalized in the cytosol, around 10% is in the mitochondria (31) and a fraction is in the endoplasmic reticulum (32). The glutathione biosynthesis pathway is depicted in Figure 1.3. Briefly, an intermediate of the methionine cycle, homocysteine, is converted into cysteine by the transsulfuration pathway, and eventually into glutathione by the glutathione synthetic pathway (30). Glutathione synthetase catalyzes the last step of the addition of glycine to γ-glutamylcysteine dipeptide. We speculate that reduced levels of glycine might have a negative impact on glutathione production. Glutathione is required for various critical functions, some of which are: (1) antioxidant defense and maintenance of redox balance; (2) detoxification of exogenous and endogenous metabolites; and (3) as a source of cysteine (27, 33, 34). All aerobic organisms generate some physiological in addition to pathological oxidative stress. The oxidative stress intermediates that are formed- hydrogen peroxide, superoxide, toxic free radicals- are reduced by glutathione peroxidase, in the process oxidizing GSH to GSSG. The GSSG formed is reduced back to GSH by glutathione reductase, completing one redox cycle. This reaction utilizes 1 molecule of NADPH (35).

1.2.4 **Glycine is required for production of creatinine**

The entire molecule of glycine is utilized in the production of creatine in turn consuming around 16% of dietary glycine (36, 37). Creatine and phosphocreatine are not only an energy buffer but also a shuttle for high-energy phosphates which are generated in the mitochondria and shuttled to the cytosol for utilization (36, 38).

Creatine and creatine phosphate are present in large amounts in vertebrate cells, like skeletal muscle and cardiomyocytes, that have high and fluctuating energy demands. There is a spontaneous loss of around 1.7% of the total body creatine pools per day which get converted irreversibly to creatinine and excreted by the urine (36, 38). This necessitates a continuous replacement of creatine pools either through dietary intake or de novo synthesis. De novo synthesis accounts for around 50% of the creatine pool (39). Creatine biosynthesis is a 2-step enzymatic process utilizing three amino acids- glycine, arginine, and methionine. The first step is the transfer of an amidino group from arginine to the amino group of glycine by the enzyme arginine:glycine aminotransferase generating guanidinoacetate and ornithine. The second reaction utilizes methyl group transfer from S-adenosylmethionine to guanidinoacetate catalyzed by guanidinoacetate methyltransferase, generating creatine and S-adenosylhomocysteine (36).

1.2.5 **Glycine functions as a neurotransmitter**

N-methyl-D-aspartate (NMDA) receptor is an excitatory (40) while the glycine receptor is an inhibitory neurotransmitter (41) in the mammalian CNS. The NMDA receptor has 2 subunits- GluN1 (activated by co-agonists glycine and d-serine) and GluN2 (activated by glutamate). GluN1 is expressed ubiquitously (42). Glycine binding to the glycine receptor opens the channel for passive diffusion of chloride ions across the membrane [reviewed in (41)]. The glycine transporters, main regulators of glycine in the vicinity of the NMDA receptors, are upregulated in obesity.

This results in increased uptake of glycine into the cell, thereby reducing extracellular glycine levels in the vicinity of NMDA receptors and therefore less potentiation of these receptors (43). In contrast, a zebrafish model of severe glycine encephalopathy, gldc -/- characterized by accumulation of glycine, showed NMDA and glycine receptor overstimulation (44).

1.2.6 **Glycine participates in conjugation and detoxification reactions**

Conjugation is a type of biotransformation system where endogenous and xenobiotic metabolites are converted to less toxic hydrophilic conjugates that can be excreted in the urine. Glycine conjugation plays an important role in this detoxification process (45, 46). The enzyme glycine N-acyltransferase catalyzes the conjugation of glycine with the xenobiotic metabolites (e.g., benzoic acid). It plays a vital role in coenzyme A homeostasis as this reaction maintains appropriate levels of free coenzyme A (46). Glycine also plays a role in the circulation of bile acids from the liver to the small intestine. Bile acids facilitate the absorption of dietary lipids, maintain cholesterol homeostasis (46), and act as signaling molecules (47). In humans, cholic acid and chenodeoxycholic acid are the primary bile acids derived from cholesterol. Secondary bile acids are produced during enterohepatic circulation. In humans, both primary and secondary bile acids are conjugated with glycine or taurine (46, 48, 49). Insulin resistance and obesity can lead to various defects in the bile acid levels or transport. Bile acid synthesis is enhanced while transport (uptake and efflux) is impaired in these metabolic syndromes (50).

In a study by Gregus, Z., et al. (1993), hepatic glycine concentration was rapidly increased to 2-3 fold normal levels by the usage of a potent inhibitor of the glycine cleavage system, Cysteamine. This led to an increase in glycine-benzoic acid conjugation and subsequent clearance from the blood by 50% and excretion of benzoyl glycine in the urine. Glycine cleavage system maintains glycine homeostasis and its activity is a significant determinant of glycine conjugation of xenobiotic metabolites (51).

1.3 The potential physiological roles of glycine degradation products

1.3.1 One-carbon groups produced by glycine degradation

One-carbon metabolism incorporates cellular nutrient status as an input in the form of carbon-units from amino acids into the folate-methionine-transsulfuration pathways and in the process generating various outputs. These outputs include, but are not limited to, redox balance, methyl group donors, and nucleotide biosynthesis (Figure 1.4). The amino acid inputs into one-carbon metabolism can be synthesized de novo (52).

A glycolysis pathway shunt at the 3-phosphoglycerate intermediate redirects carbon units from glucose towards serine biosynthesis (52). In addition to de novo synthesis, amino acid transporter, SFXN1, can also directly import serine into the mitochondria (53). Serine is further converted into glycine by the mitochondrial enzyme, serine hydroxymethyltransferase (SHMT2), in the process converting tetrahydrofolate (THF) to methylene THF (meTHF). meTHF can start the folate cycle (52). Glycine can also be directly imported into the mitochondria via the transporter, SLC25A38 (54). In some cells, glycine is cleaved into NADH, ammonia, carbon dioxide, and meTHF by the mitochondrial tetrameric glycine cleavage system. The carbon units of meTHF generated from glycine breakdown feed into the folate cycle. In rodents, the enzyme threonine dehydrogenase (TDH) can catabolize threonine to generate glycine for one-carbon metabolism and acetyl-CoA which enters the tricarboxylic acid (TCA) cycle (55).

Humans are deficient in this pathway as the human TDH enzyme has inactivating mutations (56). Some other routes of glycine synthesis are via choline, dimethylglycine, sarcosine, and betaine (52).

The biosynthetic outputs of the one-carbon metabolic pathway is synthesis of nucleic acids, proteins, and lipids that are required to support cell growth (57). Both serine and glycine via the folate cycle contribute one-carbon units required for nucleotide biosynthesis (58). Methionine, which can be used for protein synthesis (59), and S-adenosylmethionine, which is the major methyl group donor, are outputs of the methionine cycle (60). The head group of the phospholipid, phosphatidylcholine, is synthesized from choline after three methylation reactions involving S-adenosylmethionine as the methyl group donor (61, 62).

Many of the anabolic outputs from the one-carbon pathway require constant regeneration of the reducing potentials to support the biosynthetic needs. This mainly occurs through the reduction of NADPH and oxidation of $NADP^+$ (57). One of the outputs of the transsulfuration pathway is the antioxidant glutathione. Glutathione scavenges reactive oxygen species (ROS) to maintain the appropriate ratio of reducing potentials for anabolic metabolism (63). The regeneration of NADPH is required to maintain a redox balance in the intracellular environment by keeping glutathione under the reduced state (57).

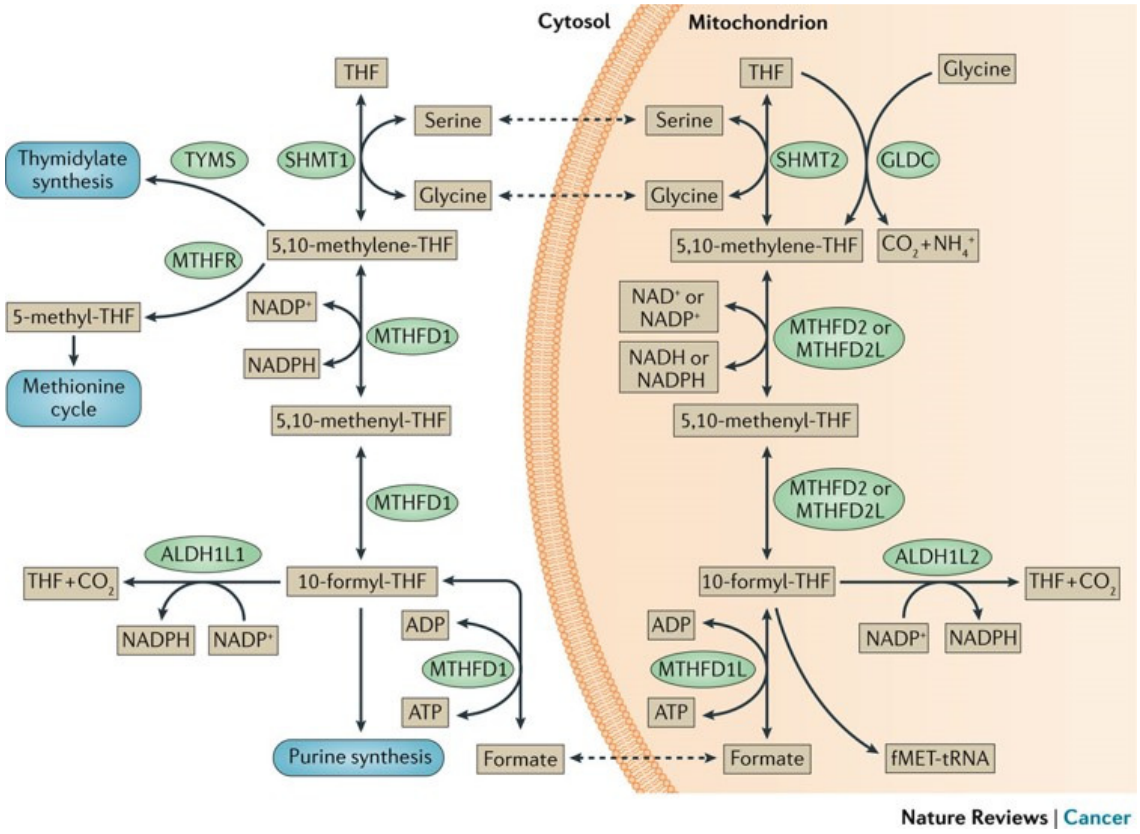

Figure 1.4: Intersection of the one-carbon metabolic pathways to support nucleotide biosynthesis and redox balance

Serine and glycine are the major contributors to the folate one-carbon units via the action of the enzymes SHMT1, SHMT2 and GLDC (64).

Another cellular requirement met from the one-carbon metabolism is the supply of substrates for signal transduction pathways via post-translational modifications. S-adenosylmethionine donates methyl groups for histone, nucleotide and protein methylation reactions necessary for many biological processes (65-68).

1.3.2 **Glycine degradation produces energetic electrons in the form of NADH**

One of the outcomes of glycine cleavage reaction is the generation of 1 molecule of NADH. Due to the proximity of the mitochondrial glycine cleavage reaction with the electron transport chain, the NADH generated can readily donate electrons for oxidative phosphorylation (69, 70).

1.4 The physiology and biochemistry of the glycine cleavage system

Most of the glycine is catabolized by the glycine cleavage system which is present in the mitochondria of some cells. This system catalyzes an oxidative cleavage reaction of glycine to generate carbon dioxide, ammonia, NADH, and a methylene group (Figure 1.5). This is a reversible reaction and the system then is termed as glycine synthase. Glycine cleavage generates one-carbon groups for the folate one-carbon metabolism pathway in the form of methylenetetrahydrofolate. Another glycine cleavage end-product is NADH which can be directly used by the mitochondrial electron transport chain to generate energy (69).

The glycine cleavage system is a complex of three enzymes and a low molecular weight carrier protein (71-73).

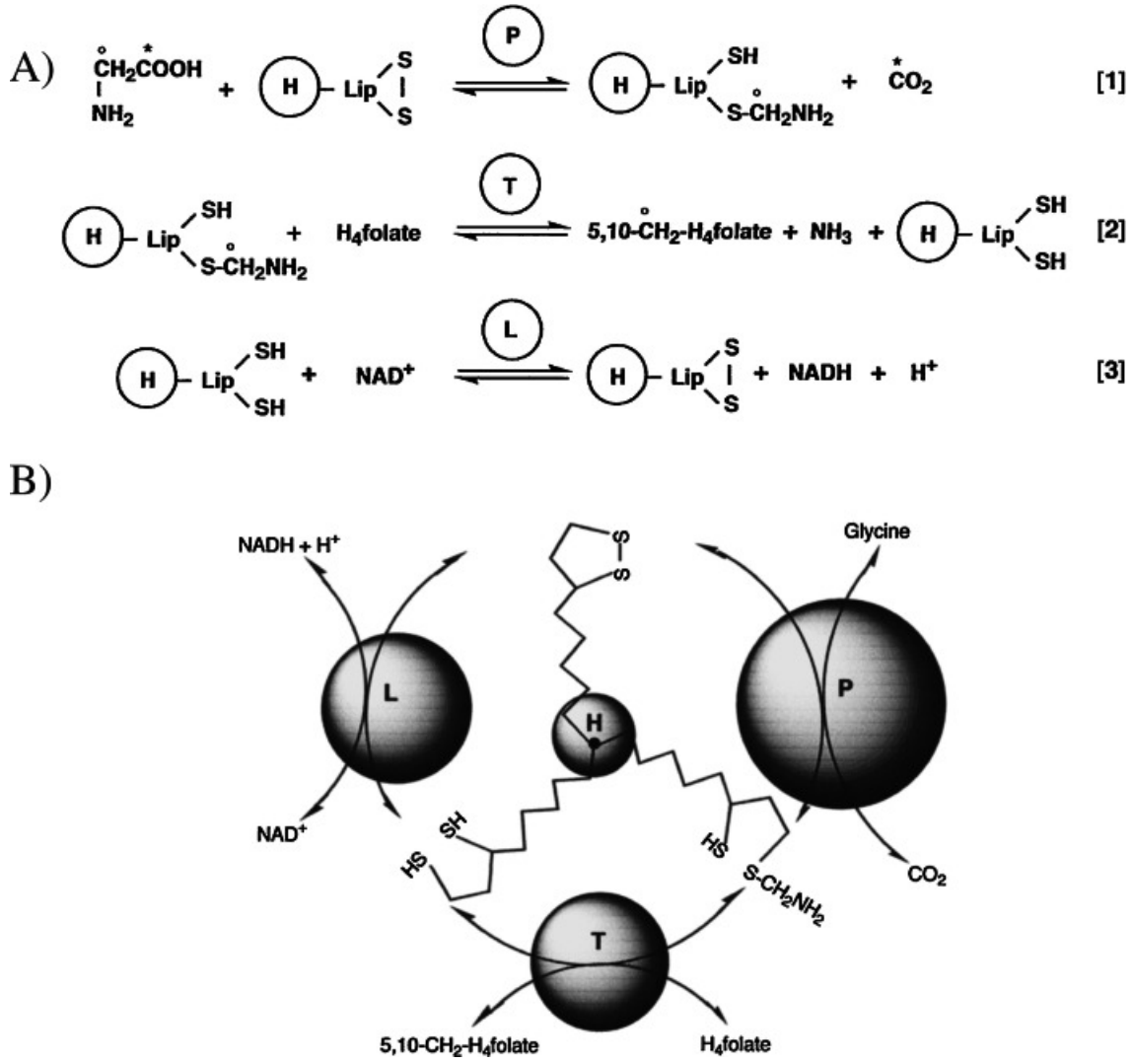

Figure 1.5: Reaction mechanism and schematic of the glcyine cleavage complex
(A) P, T, L and H represent the protein component. Lip: lipoyl moiety, H4folate: tetrahydrofolate, 5,10-CH2-H4folate: methylene tetrahydrofolate. (B) Schematic representation of the glycine cleavage complex (69).

This four-protein complex is situated closely together for the efficiency of the reactions and attached to the mitochondrial inner membrane as is the case with respiration-linked dehydrogenases like pyruvate dehydrogenase and alpha-ketoglutarate (70). The components of the glycine cleavage system are introduced in the sub-sections that follow.

1.4.1 **P-protein or glycine dehydrogenase (decarboxylating) (EC1.4.4.2) or GLDC:**

This is a pyridoxal phosphate-containing protein that catalyzes the first partial decarboxylation reaction of glycine along with a co-substrate, the carrier H-protein. In this sequential random reaction, the carboxyl carbon of glycine results in generation of carbon-dioxide (74). The intermediate is attached to the carrier protein. GLDC can exist either as a homodimer (e.g., in humans (75)) or as a dimer of heterodimers (reviewed in (69)).

1.4.2 **T-protein or Amino methyltransferase (EC2.1.2.10) or AMT:**

The second partial reaction of the decarboxylated glycine moiety which is attached to the carrier protein is carried out by AMT. The reaction requires the presence of tetrahydrofolate. This reaction results in the amino group of glycine to be released as ammonia. The second product is the methylene carbon of glycine and tetrahydrofolate being converted to 5,10-methylene tetrahydrofolate (76).

1.4.3 **L-protein or dihydrolipoamide dehydrogenase (EC1.8.1.4) or DLD:**

The previous reaction intermediate, reduced lipoate moiety attached to the H-protein, is re-oxidized by DLD as the last step of glycine cleavage. DLD is not a unique enzyme of the glycine cleavage system. It is an E3-component of 2-oxoacid dehydrogenase complexes like pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (69).

1.4.4 **H-protein or GCSH:**

This is a low molecular weight hydrogen carrier protein. The main function of the H-protein is to shuttle the glycine cleavage reaction intermediates and reducing equivalents between the active sites of the three enzymatic components of the system (71).

1.5 Deficiency or mutations in glycine cleavage system components cause nonketotic hyperglycinemia and neural tube defects

Loss-of-function mutations in any genes encoding the core components (GLDC, AMT, and GCSH) of the glycine cleavage system cause non-ketotic hyperglycinemia (NKH) or glycine encephalopathy. This is a rare autosomal recessive disorder in which glycine levels accumulate in body fluids (77, 78). Even though this is a rare disease, its occurrence is high among specific geographical areas- Israel (79), Netherlands, 1/60,000 Canada, and 1/12,000 Finland (77). More than 80% of the NKH cases are due to mutations in GLDC, the rate-limiting enzyme of the glycine cleavage system (79). Symptoms of NKH typically manifest in the first few days of life. Neonates present with progressive loss in muscle tone, lethargy, myoclonic jerks, respiratory issues, and coma. Almost one-third of NKH patients do not survive the first year.

The ones that do survive either with assisted breathing or because NKH presented later in infancy have severe neurological defects. These include seizures, hydrocephaly, and developmental disabilities (78, 80, 81).

GLDC has two significant functions:

(1) glycine decarboxylation and subsequent transfer of the amino-methyl moiety to GCSH (82); and

(2) transfer of a one-carbon group, meTHF, into the folate cycle (75, 83).

Both functions are critical for normal development and brain function. In addition to the development of NKH, a loss of glycine cleavage activity due to mutations in GLDC or AMT can also lead to neural tube defects (84). Pai, et al., (2015) generated a gene-trap based mouse model with decreased GLDC expression and absence of glycine cleavage activity. As expected, glycine levels were elevated in the plasma and urine of these mutant mice. This model showed that glycine cleavage system -activity was necessary for neural tube closure. Decreased GLDC expression in these mice led to reduced input on one-carbon units into the folate cycle and therefore reducing formate production required for cytoplasmic methylation cycle and nucleotide biosynthesis (77). When pregnant dams were provided formate supplemented drinking water from the first day of pregnancy, the folate profile of GLDC homozygous mutants was normalized and neural tube defects were rescued. But glycine levels continued to be elevated even after formate treatment. Mutations in GLDC can therefore result in two distinct phenotypes- NKH and neural tube defects (77).

1.6 GLDC is overexpressed in many cancers

GLDC is aberrantly overexpressed in some types of cancers. A study conducted by Jain et al. (2012) systematically characterized cellular metabolic activity in 60 well-established human cancer cell lines derived from nine tumor types. Out of the 111 metabolites studied, glycine levels were significantly correlated with transformed cell proliferation (specifically, increased glycine uptake by the cells from the media).

In proliferating cells, either the entire molecule of glycine can be directly incorporated into the purine ring or glycine can be further oxidized by the glycine cleavage system to provide one-carbon units for nucleotide biosynthesis and methylation reactions (85). One study found high levels of GLDC in the tumor-initiating cell in non-small cell lung cancers (NSCLC). In these cells, GLDC metabolic activity was required for maintaining the tumorigenic capacity. Overexpressing GLDC induced significant changes in glycine metabolism and glycolysis; and pyrimidine metabolism (86). Even in a tumor environment with poor vascularization, cancer cells can support their survival by altering metabolism. A study conducted by Kim, D. et al. (2015) showed glycine metabolism played a critical role in glioblastoma multiforme. GLDC expression is increased in the pseudopalisading cells surrounding necrotic foci in the ischemic zone of glioblastomas (87). In another study in phyllodes tumors (breast neoplasms), stromal expression of GLDC was positively correlated with increasing tumor grade, increased Ki-67 (proliferation marker) expression, tumor recurrence, and metastasis (88). GLDC was also seen to be highly expressed in estrogen-receptor positive breast cancer and was associated with poor prognosis. Estrogen receptor activation by estradiol was seen to upregulate GLDC in addition to the serine metabolic pathway (89).

The enrichment of the metabolite, sarcosine (derived from glycine), was identified in a prostate cancer study to have a positive association with the progression of the disease from benign to malignant and then leading to metastasis (90). In contrast to the tumorigenic potential of GLDC as shown in prostate cancer, GLDC seems to play a tumor suppressor role in gastric cancers.

GLDC gene silencing shown in this study was due to promoter hypermethylation (91). GLDC may have varied functions in different cancers and at different stages of cancer progression. Barring this one exception, together, these findings demonstrate that glycine metabolism and GLDC are involved in promoting a malignant phenotype.

1.7 GLDC is an evolutionarily conserved enzyme with highly flexible metabolic functions

In plants, as in all eukaryotes, glycine cleavage system is a mitochondrial complex. Together with SHMT, it plays a critical role in the mitochondrial component of photorespiration that converts 2-phosphoglycolate into 3-phosphoglycerate (92, 93). Overexpression of glycine cleavage system components in Arabidopsis thaliana (a C3 plant) increases flux through the photorespiratory pathway. This increases photosynthetic efficiency and the production of biomass (93). In higher plants, the glycine cleavage system is indispensable for both photorespiration and critical metabolic processes via participation in one-carbon metabolism (92, 94, 95).

The fact that the glycine cleavage system in general and GLDC in particular plays such fundamental and varied roles in organismal physiology; from growth regulation (overexpression of GLDC in cancer cells) to photosynthesis in plant cells, together with its highly evolutionary conservation from bacteria (96, 97) to humans suggests that this pathway is deeply integrated in many basic metabolic pathways.

1.8 The objective of the dissertation, hypothesis, and specific aims

The overall objective of my thesis work is to improve our understanding of the regulation and physiological role of glycine decarboxylase (GLDC), the rate-limiting enzyme in the degradation of glycine. Multiple metabolomic studies have previously shown that circulating glycine levels are reduced in patients and animals with type 2 diabetes and related metabolic conditions, insulin resistance, obesity, and NAFLD. Although these are purely correlative findings, the diverse metabolic roles of glycine suggest potential functional links between glycine metabolism and these disorders. One of these is especially interesting in the context of diabetes; glycine is a required substrate for the biosynthesis of glutathione, which is required for defense against cellular oxidative stress.

Increased oxidative stress is a hallmark of many metabolic disorders, including diabetes. Together, these observations raise the possibility that changes in glycine metabolism, mediated by altered GLDC expression, could have an impact on susceptibility or progression of diabetes and related metabolic disorders. As a basis for my exploration of this possibility we have formulated the following hypotheses:

(1) GLDC, the rate-limiting enzyme of the glycine cleavage system is upregulated in metabolic disorders or metabolic stress,

(2) Regulation of GLDC expression is integrated with the hormonal signaling system that maintains metabolic homeostasis,

(3) Alteration of GLDC expression affects cellular redox balance via changes in glutathione production.

The specific aims of my thesis work are:

Aim 1: Determine if GLDC gene expression is altered in a disease state or under metabolic stress.

Aim 2: Identify the metabolic or hormonal signals that regulate gene expression and characterize the transcriptional pathways that mediate this regulation.

Aim 3: Determine the impact of altered GLDC expression on glutathione production and cellular redox status.

CHAPTER 2 - EXPRESSION OF GLYCINE DECARBOXYLASE (GLDC) IN RODENT MODELS OF METABOLIC DISORDERS AND NUTRITIONAL STRESS

2.1 Introduction

The observation that glycine levels are reduced in diabetes and related metabolic conditions forms the scientific premise of my thesis project. We hypothesized that the relationship between metabolic status and the level of circulating glycine is dictated by changes in the amount or activity of GLDC, the rate limiting enzyme for glycine degradation (see the Background section for more detail). To determine if GLDC gene expression was altered by metabolic stress we used mouse models of human metabolic diseases. We focused on hepatic GLDC gene expression as the liver is the primary site for glycine degradation in the body and has a major role in determining circulating glyine levels (98).

We examined hepatic GLDC gene expression in both genetic and diet-induced models of diabetes. While the rodent models of diabetes do not perfectly mimic all aspects of the human disease, (e.g. pancreatic islet pathology), they are excellent models for many of the broad pathophysiological characteristics seen in the human disease (99, 100).

The db/db mouse has a genetic leptin-receptor deficiency, which results in hyperphagia, hyperinsulinemia (by 2 weeks of age), elevated glucagon levels, obesity (by 4 weeks of age), and hyperglycemia with beta-cell disruption (between 4 to 8 weeks) (100) (101). The ob/ob mice have mutations in the leptin gene which also results in hyperphagia and obesity (by 4 weeks of age) along with mild hyperglycemia, compensatory hyperinsulinemia, and insulin resistance (100). The diet-induced C57BL6/J mouse model utilizes a high-fat diet to induce obesity and a diabetes-like state.

Mice given 65% calories from fat diet for 15 weeks develop hyperinsulinemia, elevated fasting blood glucose, insulin resistance, and glucose intolerance (102). In addition to these models of metabolic stress via caloric excess, we also examined hepatic GLDC gene expression in response to nutritional stress - fasting.

The specific goals of the experiments described in this chapter are to measure GLDC gene expression at the RNA and protein level, under the disease models described above. If our hypothesis is correct, we should observe an elevated level of hepatic GLDC gene expression in the diabetes and obesity models described above. Changes in GLDC expression in these metabolic stress models would guide us in examining the the hormonal signals that regulate GLDC gene expression.

2.2 Materials and methods

2.2.1 **Animal experiments**

All the animal experiments were approved by the Wayne State University IACUC committee and carried out under the institutional guidelines for ethical animal use.

2.2.2 **A genetic model of type II diabetes**

The leptin receptor-deficient db/db and control db/+ mice livers were a gift from Dr. Jeimei Wang, Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI. The livers were from 9-week-old, male mice. These mice were not given any special diet. The control db/+ mice were age- and gender-matched healthy litters from the same colony. The ob/ob mice data was obtained from the NCBI Gene Expression Omnibus (GEO) public database (National Center for Biotechnology Information, Bethesda, MD, USA; https:// www. ncbi. nlm. nih. gov/ geo/). The raw microarray dataset (GEO profile GDS4506) was from 3-month-old, male ob/ob mice on lab diet 5058. Their livers were harvested after an overnight fast.

2.2.3 **Diet-induced obesity mouse models**

Male C57BL6/J mice were either given a low fat or a high-fat diet for 16 weeks to induce insulin resistance and generate a diet-induced obesity mouse model. The low-fat diet provided 10% while the high-fat diet provided 45% calories from fat. At the end of the study duration, livers were harvested and snap-frozen for analysis by immunoblotting.

2.2.4 **Fasting**

Male C57BL6/J mice were either fasted for a short-term (6h), a physiologic (12h), and extended duration (16h and 18h). All mice had unrestricted access to water. The control mice had ab/lib access to normal chow. At the start of the fasting duration, the mice were moved to a clean cage without food. The fast was started in such a way that all time points (controls and fasted groups) were terminated at the same time. Livers were harvested and snap-frozen for analysis.

2.2.5 **RNA isolation and quantification**

Liver tissue (50-100 mg) was lysed and homogenized in 1 ml TRIzol (Invitrogen) using a tissue ruptor II (Qiagen) apparatus. The samples were centrifuged at 12000 X g for 5 min at 4°C to get rid of the non-homogenized tissue material and any high-fat content, if present. RNA isolation using TRIzol was carried out based on the manufacturer's protocol. For RNA isolation from cultured cells, total RNA was isolated after treatment with hormones using PureLink RNA mini kit (Invitrogen). The total RNA isolated was quantified by absorbance spectrophotometry using a Nanodrop. Only samples with total RNA A260/230 ratio of 2.0-2.2 were used for cDNA synthesis.

2.2.6 **Reverse transcription and cDNA synthesis**

Total RNA was reverse transcribed to cDNA according to the reaction setup recommendations of the manufacturer (Applied Biosystems). The random primers used in this kit ensure that the first strand synthesis takes place efficiently with all species of RNA

molecules present, including mRNA and rRNA. In short, the 2X RT master mix was added in 1:1 ratio to 1500 ng RNA sample per PCR tube.

The thermal cycler conditions were set as follows:

Settings	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temp.	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	Hold

2.2.7 **Quantitative real-time polymerase chain reaction**

For real-time PCR analysis, reaction mixtures containing cDNA template, primers, and SYBR Green PCR Master Mix (Thermo Scientific) were analyzed with Stratagene MX3000P (Applied Biosystems, Carlsbad, CA). The transcript abundance of the different genes tested was quantified after normalization to internal control PPIA. Specific oligonucleotides, spanning an exon-exon junction, were designed using Primer-BLAST (NCBI) and Oligoanalyzer3.1 (Integrated DNA technologies). The primers were designed such that amplicon size would be between 70 and 200 bp. The 3' end of the primers selected had either a C or G residue to minimize non-specific binding to DNA and a GC content of approximately 40-60% for maximum product stability. The sequences of the real-time PCR primers used are listed in Table 2.1

The real-time PCR program protocol included an initial activation at 95 °C for 15 minutes, followed by 40 cycles of melting with a denaturation step at 95 °C for 20 sec, annealing at 58 °C for 25 sec and extension at 72 °C for 25 sec per cycle. The real-time PCR was performed in duplicates and the relative fold changes were quantified using the $2^{-\Delta\Delta Ct}$ method. Results are expressed as mean $\pm$ S.D.

Table 2.1: Primers used in the RT-qPCR analysis

Primer Name	**Sequence**
Mouse GLDC forward	CATGATTGAGCCCACCGAGT
Mouse GLDC reverse	TGGAGACATCTTCAGGGGGT
Mouse FASN forward	GACTCGGCTACTGACACGAC
Mouse FASN reverse	CGAGTTGAGCTGGGTTAGGG
Mouse PCK1 forward	GTTTGATGCCCAAGGCAACT
Mouse PCK1 reverse	CCTTCCCAGTAAACACCCCC
Mouse PPIA forward	GCGTCTCCTTCGAGCTGTT
Mouse PPIA reverse	CTCTCCGTAGATGGACCTGC

2.2.8 **Western blot analysis using liver tissue**

Samples of liver tissue (50-100 mg) were homogenized or lysed in 500 µl RIPA lysis buffer (Thermo scientific) and freshly added protease inhibitor cocktails using a tissue homogenizer. After complete homogenization, the tissue was centrifuged at 14000 X g for 20 min at 4 °C. The clear supernatant was carefully aspired and transferred to a new microcentrifuge tube. For isolating protein from cells, cells were washed twice with ice-cold PBS and then collected in PBS using a cell lifter. Cells were pelleted at 10000 rpm for 10s at 4°C. Supernatant was aspirated and 100 µl RIPA lysis buffer (Thermo scientific) along with fresh protease inhibitor cocktails was added to the cells and vortexed for 20 sec. The lysate was sonicated at 20% amplitude and pulse for 45 sec on-30 sec off- 45 sec on ice. Protein concentrations were determined with a bicinchoninic (BCA) protein assay kit according to the manufacturer's instruction (Pierce Biotechnology) and read on a CLARIOStar plate reader at 562nm. 40 µg of protein was mixed with SDS-PAGE loading dye and the samples were heated at 99 °C for 5 min. The samples were then loaded on a 10% SDS-PAGE gel. After separating the proteins based on molecular weights, the proteins were then transferred onto a nitrocellulose membrane.

The membrane was then incubated in 5% non-fat milk in PBS-T buffer at room temperature for 2h to block non-specific binding. The blots were then probed with specific primary antibodies at 4°C overnight. Primary antibodies used GLDC (Sigma), GAPDH (Santa Cruz), and Actin (Sigma). Primary antibodies were diluted 1:1000 in 5% non-fat milk in PBS-T. Following incubation, the blots were washed 3 times for 10 min each with 1X PBST to remove the unbound primary antibody. The blots were then incubated with HRP-conjugated secondary antibody at room temperature for 1-2h. Following incubation, the blots were washed 3 times for 10 min each with 1X PBST to get rid of the unbound secondary antibody. The protein bands were visualized with enhanced chemiluminescence reagents (Perkin Elmer).

2.2.9 **Statistical methods**

Statistical comparisons were made with the Student's t-test. $P<0.05$ was considered significantly different. Data are presented as means $\pm$ S.D. In each experiment, all treatments were performed in triplicate. All statistical analysis was performed using GraphPad Prism software (La Jolla, CA).

2.3 Results

2.3.1 **Hepatic GLDC expression is upregulated in a mouse model of type 2 diabetes**

To determine whether GLDC expression is modulated by glycemia and/or insulinemia, we used the leptin receptor deficient db/db mouse which is routinely used as a model for type II diabetes (99, 100). We used chow fed 9-week-old male mice. Age matched male db/+ litermates were used as controls. As shown in Figure 2.1 A, hepatic GLDC mRNA levels were significantly elevated (2-fold, $P=0.028$) in the db/db mice as compared to db/+ controls. As a positive control for diabetes-mediated changes in gene expression we also measured the lipogenic gene fatty acid synthase (FASN), which as expected was significantly upregulated (3.63-fold, $P=0.0289$) in db/db as compared to db/+ mice (Figure 2.1 B). These findings are consistent with profiling data from ob/ob mice (extracted from GEO profiles) (Figure 2.1 C).

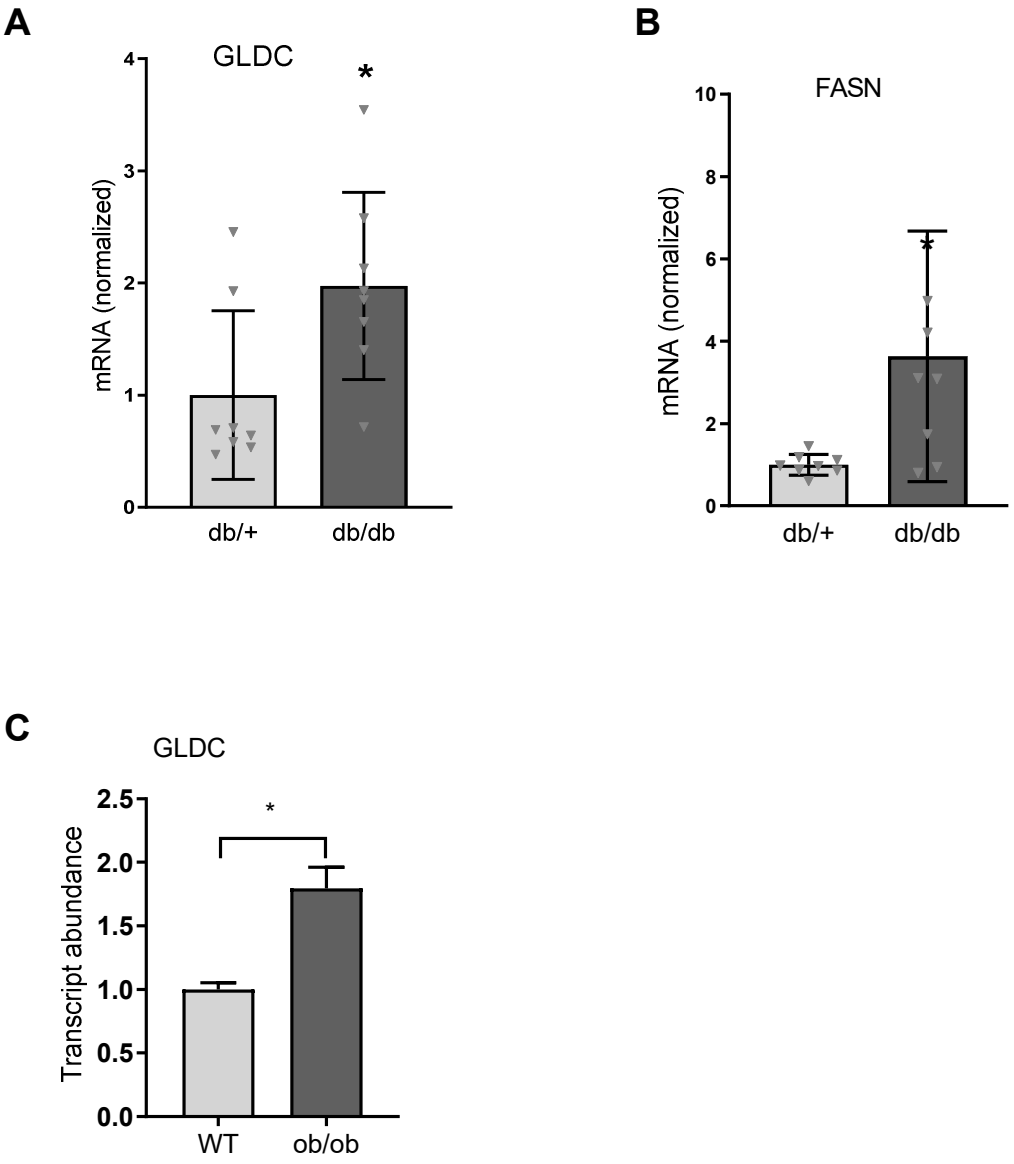

Figure 2.1: Hepatic GLDC is upregulated in a mouse model of type 2 diabetes
(A) Hepatic GLDC and (B) hepatic FASN mRNA of 9-week-old db/+ (n=8) vs db/db (n=8) male mice on no special diet. Transcript abundance was analyzed using $2^{\Delta\Delta Ct}$ method and quantitated by RT-qPCR. TBP was used as a reference gene. Error bars, mean $\pm$s.d.; *P<0.05. . (C) The polysome-bound GLDC transcripts in the endoplasmic reticulum from livers of WT (n=2) and ob/ob (n=2) mice. Data was extracted and plotted from GEO profiles (GEO accession number: GSE39375 series).

2.3.2 **Hepatic GLDC is elevated in a diet-induced obesity mouse model**

A high-fat diet (more than 30% of total energy from fat) for 12-16 weeks is used to induce obesity in animal models (102). To determine if hepatic GLDC expression is elevated in a diet-induced obesity mouse model we fed C57BL6/J mice either a low-fat control diet (10% calories from fat) or a high-fat (45% calories from fat) diet, ad lib, for 16 weeks to induce insulin resistance. GLDC protein levels were moderately elevated in the livers of the mice fed a high-fat diet compared to the lean control mice (Figure 2.2 A and B). Although not statistically significant, there was a tendency towards elevation in GLDC protein levels in the livers of the mice on a high-fat diet. Together these results demonstrate that GLDC expression is elevated in type II diabetes and diet-induced obesity models.

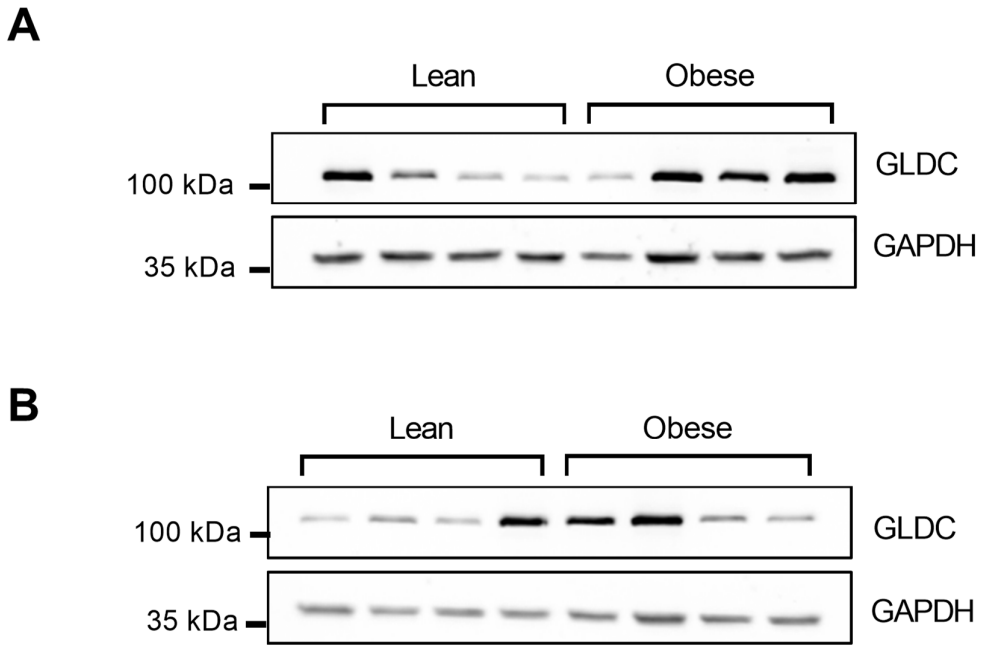

Figure 2.2: Hepatic GLDC expression in diet-induced obesity mouse model
Western blot of GLDC expression in livers from C57BL6/J mice in (A) 'Group 1' lean (n=4) and obese (n=4) mice and (B)) 'Group 2' lean (n=4) and obese (n=4), fed a low fat (LF, 10% calories from fat, n=8) and high fat (HF, 45% calories from fat, n=8) diet for 16 weeks. GAPDH was used as a loading control.

To further support our findings, we examined the NCBI Gene Expression Omnibus (GEO) public database (National Center for Biotechnology Information, Bethesda, MD, USA; https:// www. ncbi. nlm. nih. gov/ geo/) for studies on hepatic GLDC regulation. Findings from a circadian biology study in mice (accession number: GSE52333 series) (103, 104) showed that C57BL6/J mice fed either a normal chow or a high-fat (60% calories from fat) diet for 10 weeks displayed significantly elevated hepatic GLDC mRNA abundance at ZT=20 (Figure 2.3 A). It is noteworthy that the high-fat diet did not affect the GLDC rhythmicity. We also looked at the effect of high fat diet on the expression of the other components of the glycine cleavage system (AMT, DLD and GCSH). AMT also showed elevated expression in animals fed a high fat diet (Figure 2.3 B)

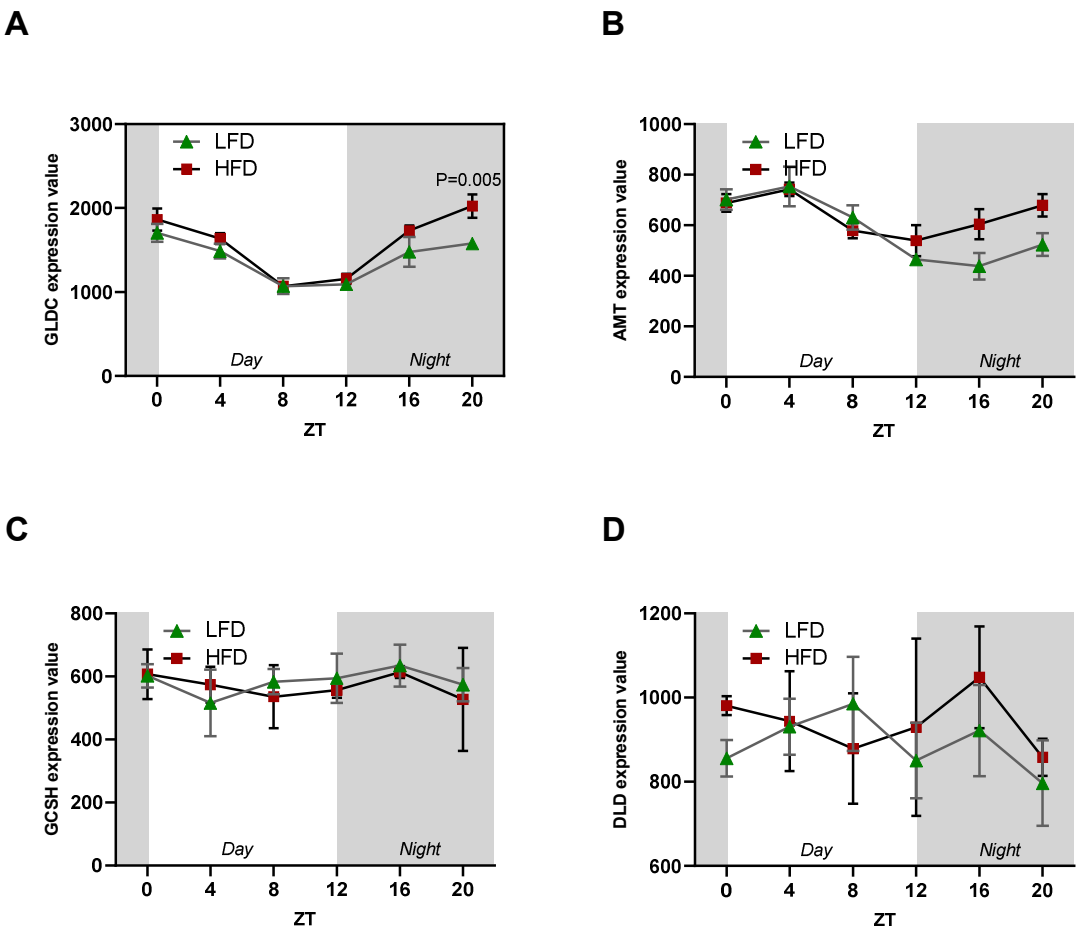

Figure 2.3: Circadian rhythmicity in the transcript abundance of hepatic glycine cleavage system components in a mouse model of diet-induced obesity

Hepatic mRNA analysis of the core components (A) GLDC, (B) AMT, (C) GCSH, and the component shared with other pathways (D) DLD; in livers of C57BL6/J mice after 10 weeks on a high-fat diet. Livers were harvested every 4 hrs throughout the 24 hr Zeitgeber time (ZT). Microarray data has been extracted and plotted from GEO profile (accession number: GSE52333 series).

2.3.3 **Hepatic GLDC is elevated during fasting**

Next, we wanted to determine if the nutritional stress associated with fasting could affect GLDC expression. We examined the effect of varying lengths of fasting duration on hepatic GLDC expression. As shown in the figure, hepatic GLDC mRNA was significantly stimulated with the 12h and 16h fast (Figure 2.4 A). As expected, fasting-induced gluconeogenic gene phosphoenolpyruvate carboxykinase (PCK1) (Figure 2.4 C) but repressed the lipogenic gene fatty acid synthase (FASN) (figure 2.4 B). Hepatic GLDC protein levels did not increase during the short or extended fasting duration but PCK1 protein levels were stimulated at 6h and 12h (data not shown). These results suggest that the hormonal signals or nutritional environment associated with fasting regulate hepatic GLDC gene expression.

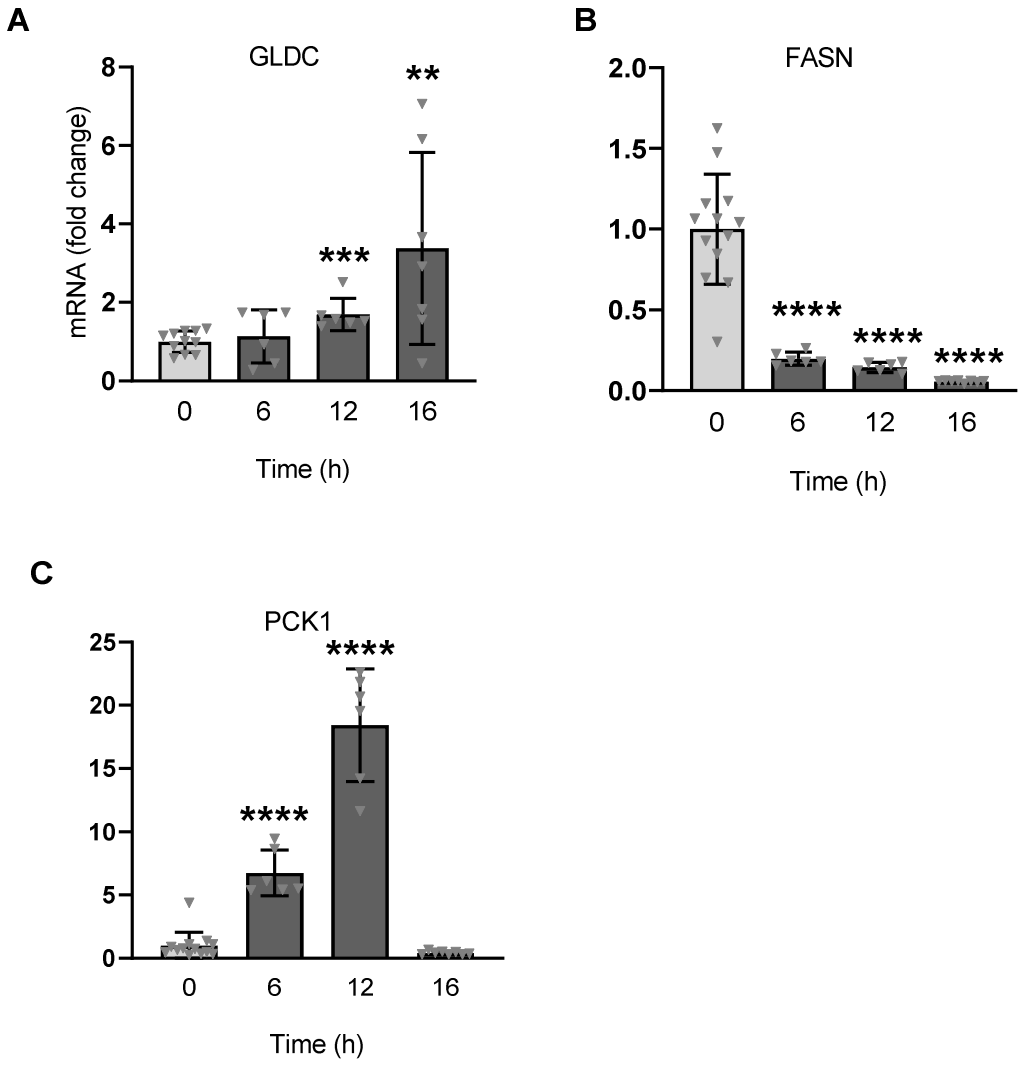

Figure 2.4: Hepatic GLDC mRNA is upregulated under nutritional stress
C57BL6/J mice were either fed ad lib (0h) or fasted for 6, 12 and 16h and hepatic mRNA abundance of (A) GLDC, (B) PCK1, and (C) FASN was quantitated by RT-qPCR. PPIB was used as a reference gene. Error bars, mean $\pm$ s.d.; * significantly different from 0h control. **P<0.01, ***P<0.001, ****P<0.0001 Student's t test.

2.4 Discussion

The goal of the experiments described in this chapter was to determine whether hepatic GLDC gene epression is altered by metabolic disease or nutrient deprivation. This is relevant because we initially proposed that the consistent observation of reduced plasma glycine levels in metabolic diseases was due to increased glycine degradation by GLDC. We examined GLDC mRNA and protein levels in several standard mouse models of metabolic disease and nutrional stress, as well as in publicly available data from GEO profiles. The results presented here are consistent with they hypothesis that GLDC overexpression contributes to the reduction in blood glycine levels seen in metabolic disease.

GLDC mRNA was significantly upregulated in the db/db and ob/ob mouse models, both of which are characterized by hyperinsulinemia and/or hyperglucagonemia depending on their age. This raises the possibility that the altered expression of GLDC in these metabolic conditions is mediated by either one or both of these hormones. This possibility will be explored in the next chapter.

We were intrigued by the ancillary observation that GLDC mRNA levels exhibit a circadian pattern of expression. Through a combination of synthetic data and pathway activity analysis of transcriptional profiling of rat liver, GLDC was identified as one of the genes in the glycine, serine and threonine metabolic pathway to exhibit a circadian patthern of expression (105). Many genes involved in metabolism are known to have altered rhythmicity (phase shift, inverted, blunted) in their expression patterns on a high-fat diet (106). GLDC appears to be an exception to this as hepatic GLDC transcripts maintained circadian rhythmicity even on high-fat diet. Examination of other genes in the same GEO data set showed that another core protein of the glycine cleavage system, AMT, had a similar pattern of expression. These observations suggests that the rate of glycine degradation and possibly the level of glycine in circulation

exhibit circadian fluctuations. The physiological significance of circadian fluctuations of glycine levels is unknown.

Our initial hypothesis was based on large-scale metabolomic studies done in diabetic and obese humans that observed a reduction in plasma glycine levels in the disease state. This phenomenon also occurs in mice, as shown by Eckel-Mahan, et al. (2013), who showed that high fat fed mice displayed significantly lower plasma glycine levels compared to chow fed mice (supplementary data in (103)). Our finding, taken together with these observations from other studies are strongly supportive of the our hypothesis that an overexpression of hepatic GLDC contributes to the reduction in circulating glycine levels seen in type 2 diabetes and obesity.

All these models are products of nutritional 'excess'. We used fasting as a metabolic paradigm for nutritional 'stress'. Hepatic GLDC expression was elevated with 12h, 16h and 18h of fasting. An 18h fast is a standard procedure in mice (reviewed here (107)). Fasting induces an increase in circulating glucagon, glucocorticoids, epinephrine and a decrease in insulin concentrations (107). These findings, taken together with the observation that in the disease models we examined exhibit hyperglucagonemia, suggest that glucagon may be a significant hormonal regulator of GLDC. This idea is consistent with earlier studies showing that the flux of the glycine cleavage system is stimulated by glucagon, epinephrine, vasopressin and a high-protein meal. The next chapter explores the hormonal regulation of GLDC with these hormones and the transcription factors mediating the effect of these metabolic hormones on GLDC transcription.

CHAPTER 3 - HORMONAL REGULATION OF GLDC GENE EXPRESSION

3.1 Introduction

The results described in the previous chapter indicated that hepatic GLDC gene expression in mice is altered in obesity-induced diabetes and by fasting. Obesity and type 2 diabetes are accompanied by a variety of hormonal abnormalities. Depending on the stage of diabetes development the hormonal profile can include hyperglucagonemia and hyperinsulinemia, both of which contribute to the hyperglycemia seen in diabetic patients by enhancing hepatic glucose production and reducing peripheral glucose uptake (108-112). During a fast, glucagon and glucocorticoids are elevated and act to stimulate hepatic gluconeogenesis (111, 113, 114). These observations, together with our findings on hepatic GLDC expression raise the possibility that the expression of the GLDC gene is regulated by these metabolic hormones. In this chapter, we have characterized the hormones and the signaling pathways regulating GLDC gene transcription using primary hepatocytes and hepatoma cell models.

Primary hepatocytes are an excellent experimental system for the study of the mechanism of gene expression. In this cell model, hormonal conditions can be carefully monitored and controlled to avoid the homeostatic mechanisms that are evoked by injection of the hormone directly into a living animal. Furthermore, longer duration studies can be performed in these cells, which are not possible with perfused livers, for example, for studying changes in gene expression. Primary hepatocytes more closely resemble liver cells (115). However, they do show batch to batch variability in function and viability, they do not replicate and have a relatively short lifespan. This makes it difficult to use them as a routine

experimental system. To overcome some of these difficulties, we also used some hepatoma cell lines for our studies eg. Huh7 (human), H4IIe (rat), and HepG2 (human; which was used for experiments shown in 'chapter 4'), which retain some liver-specific expression (116-118).

3.2 Materials and methods

3.2.1 **Cell culture**

H4IIE (ATCC), Huh7, HEK293A (Invitrogen), and HEK293T (ATCC) cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all purchased from Life Technologies) and 10% fetal bovine serum (FBS) (Denville Scientific). Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

3.2.2 **Primary culture of rat hepatocytes**

All procedures were performed according to the regulatory guidelines of Wayne State University division of laboratory animal resources (DLAR), Detroit MI. Adult male Sprague Dawley rats (180-200 gm) were housed in a DLAR facility with controlled temperature (23 °C) and humidity with a 12h light/dark cycle and ad-lib access to regular chow and distilled water. After receiving the animals, they were acclimatized for 1 week before use.

Primary hepatocytes were isolated by Mary Gargano using a two-step collagen perfusion method as described in detail elsewhere (Kocarek and Reddy, 1996). Immediately after isolation, primary hepatocytes were plated onto collagen type I (Advanced BioMatrix) coated tissue culture plates and cultured in Williams' E medium (Gibco, A12176-01) supplemented with 10% FBS (Denville), 100 nM Novolin (Novo Nordisk), 0.1 µM triamcinolone acetonide (Sigma), 0.02 M glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all purchased from Life Technologies) [Plating medium]. 6h after plating, the hepatocytes were overlaid with Matrigel (Corning) diluted 1:50 in Williams' E [Maintenance]

medium supplemented with 100 nM Novolin, 0.1 µM triamcinolone acetonide, 0.02 M glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

The next day, hepatocytes were renewed with the addition of fresh Williams' E maintenance media and allowed to attach and recover from the stress of the isolation procedure. 48h after isolation, treatments were applied to the hepatocytes in Williams' E [treatment] media supplemented with 0.02 M glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The medium containing the treatments was replenished every 24h as required.

3.2.3 **Transient transfections and dual-luciferase reporter assay**

Approximately 100,000 Huh7 cells/well in 0.5 ml of supplemented DMEM were plated into 24-well plates overnight. The next day, cells were transfected with a complex containing 1.5 µl of Lipofectamine 2000, 75 ng of either GLDC 1kb firefly luciferase reporter plasmid or 245 bp FASN firefly luciferase reporter plasmid, 75 ng of pBlueScript, 50 ng EGFP-N1, 300 ng pcDNA3.1-Srebp1c expression plasmid (Addgene), and 0.5 ng pRL-CMV (Promega) diluted in 100 µl Opti-MEM (Life Technologies) per well. 6h later, fresh media was added on the cells. 42h after transfection, media was changed to serum-free DMEM for 6h, then cells were lysed and collected and firefly and Renilla luciferase activities were measured using Dual-luciferase reporter assay system (Promega) on a Clariostar plate reader (BMG LabTech). For each sample, the firefly luciferase value was normalized to the corresponding Renilla luciferase value. For transient transfection of HEK293T, 800 ng pcDNA3.1-Srebp1c, 100 ng pcDNA3.1, and 100 ng EGFP-N1 were transfected using lipofectamine 2000. Media changes and cell harvesting were done as explained above. Changes in GLDC mRNA were measured by real-time PCR.

3.2.4 **siRNA-knockdown experiments**

Primary cultures of rat hepatocytes were transiently transfected with siRNA against GLDC, CREB1, and ATF1 (Dharmacon). Hepatocytes were plated on collagen-coated12-well

plates (6 X 10^5 cells per well). Cells were transfected with Metafectene Pro (Biontex Laboratories GmbH, Munich, Germany) according to the manufacturer's instructions. 6h after plating, the plating media was replaced with maintenance media containing the transfection complexes. After overnight incubation of the complexes on the cells, the culture media was replaced with fresh maintenance media containing Matrigel matrix (1:50 dilution), and the cells were incubated for 24h. The next day, fresh maintenance media was replenished on the cells. 48h after transfection of the cells, dibutyryl cAMP treatment was applied via William's E treatment media for 12h and the cells were harvested for RNA isolation. Each treatment was performed in triplicates and repeated at least twice using isolated hepatocytes from one rat for each independent experiment.

3.2.5 **The quantitative reverse-transcription polymerase chain reaction in primary cultured rat hepatocytes**

Hepatocytes (1.6 million per well) were plated immediately after isolation onto collagen-coated 6-well plates and cultured as described above. 48h after plating, hepatocytes were treated with either dibutyryl cAMP, glucagon, and/or insulin (stocks prepared in water) at concentrations and for time durations indicated in the individual figure legends. After treatment durations were complete, cells were harvested and total RNA was extracted using PureLink RNA mini kit (Invitrogen). cDNA was synthesized from total RNA using the high capacity reverse transcription kit (Applied Biosystems) using the manufacturer's protocol.

Gene-specific primer pairs were designed and analyzed for specificity using the NCBI primer-BLAST tool and purchased from Integrated DNA Technologies (IDT, Coralville, IA).

The sequences of the primers used to detect the various genes are listed in Table 3.1. PPIB was used as a reference gene. Quantitative determination of gene expression in response to the treatments was performed by real-time PCR using Sybr green low ROX master mix (Thermo Fisher), gene-specific primers (70 nM), and cDNA (75 ng) per reaction. Assays were

performed in experimental duplicates on a Stratagene Mx 3000P, and the relative mRNA fold changes were quantified using the comparative cycle threshold 2ΔΔCt method. Results were normalized to controls and are expressed as mean $\pm$ SD.

Table 3.1: Primers used in the RT-qPCR analysis

Primer Name	Sequence
Rat GLDC forward	GGAGGGGTCTTCTAGGGTCT
Rat GLDC reverse	CAGGTGATGGGCAGTGGAAT
Rat AMT forward	GGACTTCCCAGGAGCCAAAA
Rat AMT reverse	GGAGCCCCTTCGCATATCAA
Rat DLD forward	AGGTGAAAGCCCTGGTGTAG
Rat DLD reverse	GCCGATTGAAATGGCCTTTCTTG
Rat FASN forward	TCGACTTCAAAGGACCCAGC
Rat FASN reverse	ACTGCACAGAGGTGTTAGGC
Rat PCK1 forward	CCCAAGAGCAGAGAGACACC
Rat PCK1 reverse	CATACATGGTGCGGCCTTTC
Rat TBP forward	CCCCGGTGGAAGACAGTTTTA
Rat TBP reverse	CCAAGCCCTGAGCATAAGGT
Human PPIA forward	GTCTCCTTTGAGGTAAGGGGC
Human PPIA reverse	GCTGCACGATCAGGGGTAA
Human GLDC forward	GGCCCATCGGAGTGAAGAAA
Human GLDC reverse	TATCGCAGTTTCCGTGGCTT
Human SREBF1 forward	CTAGGAAGGGCCGTACGAGG
Human SREBF1 reverse	GCCGACTTCACCTTCGATGTC
Rat PPIB forward	TCCGTGGCCAACGATAAGAAG
Rat PPIB reverse	GCCAAATCCTTTCTCTCCTGTAGC
Rat GCSH forward	GAGTGGTAAGGAGTGTGCGG
Rat GCSH reverse	TTACGCACCGAGAGCAAAGC
Rat CREB1 isoA forward	TGAAGAACAGGGAAGCAGCAAGAG
Rat CREB1 isoA reverse	TTGGTTTTTAAGCACTGCCACTCTG
Rat CREB1 isoB forward	ACTGAGGAGCTTGTACCACCG
Rat CREB1 isoB reverse	CTGGCATGGATACCTGGGCT
Rat ATF1 forward	ATCCCAGCATTTCTGCCGTC
Rat ATF1 reverse	ATGGCAATGTACTGTCCGCTG

3.2.6 **Western blot analysis from cultured cells**

Cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS), scraped off the plates, and lysed in RIPA lysis and extraction buffer (Thermo Scientific) with protease inhibitors followed by sonication for 30 seconds. Protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Scientific). Cell lysates (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk in PBS, pH 7.4, containing 0.1% Tween-20 (PBST). The protein bands were visualized with enhanced chemiluminescence reagents (Perkin Elmer). Primary antibodies were used at 1:1000 dilution in 5% non-fat milk in PBST: GLDC (Sigma-Aldrich, HPA002318), AMT (Sigma-Aldrich), DLD (Sigma-Aldrich), GAPDH (Santa Cruz) and Actin (Sigma-Aldrich). The primary antibodies phospho-CREB (Cell Signaling Technology, #9191S) and total CREB (Cell Signaling Technology, #4820S) were diluted 1:1000 in 5% BSA in TBST. The signal was visualized using Azure c600 imaging system (Azure Biosystems).

3.2.7 **Statistical methods**

Data were subjected to analysis of variance, and statistical comparisons were made with the Student's t-test. $P<0.05$ was considered significantly different. Data are presented as means + S.D. In each experiment, all treatments were performed in triplicate. All statistical analysis was performed using GraphPad Prism software (La Jolla, CA).

3.3 Results

3.3.1 **Hormonal regulation of GLDC**

As mentioned above the stimulation of the GLDC gene expression by metabolic stress (type 2 diabetes and diet-induced obesity mouse models) and nutritional stress (fasting), suggested that hormones known to be altered in these conditions may be direct regulators of GLDC expression. To examine this possibility we performed an exploratory survey with the classic metabolic hormones: 8-br cAMP (as an experimental stand-in for glucagon), insulin, and dexamethasone (an analog for cortisol). We performed these experiments in H4IIE cells because of their known responsiveness to metabolic stimuli at the gene expression level. The panel is comprised of individual or combinations of hormonal treatments. As seen in Figure 3.1A, GLDC mRNA abundance was significantly upregulated with insulin and insulin + dexamethasone. We confirmed the proper hormonal responsiveness of the cells by measuring the mRNA abundance of PCK1 and FASN which are well characterized gluconeogenic and lipogenic genes, respectively. As expected PCK1 mRNA was significantly suppressed by insulin and significantly upregulated by cAMP, dexamethasone, and cAMP + dexamethasone (Figure 3.1B). FASN mRNA was significantly upregulated with insulin, dexamethasone, and insulin + dexamethasone (Figure 3.1C). These results demonstrate that insulin and dexamethasone regulate GLDC gene expression in H4IIE cells.

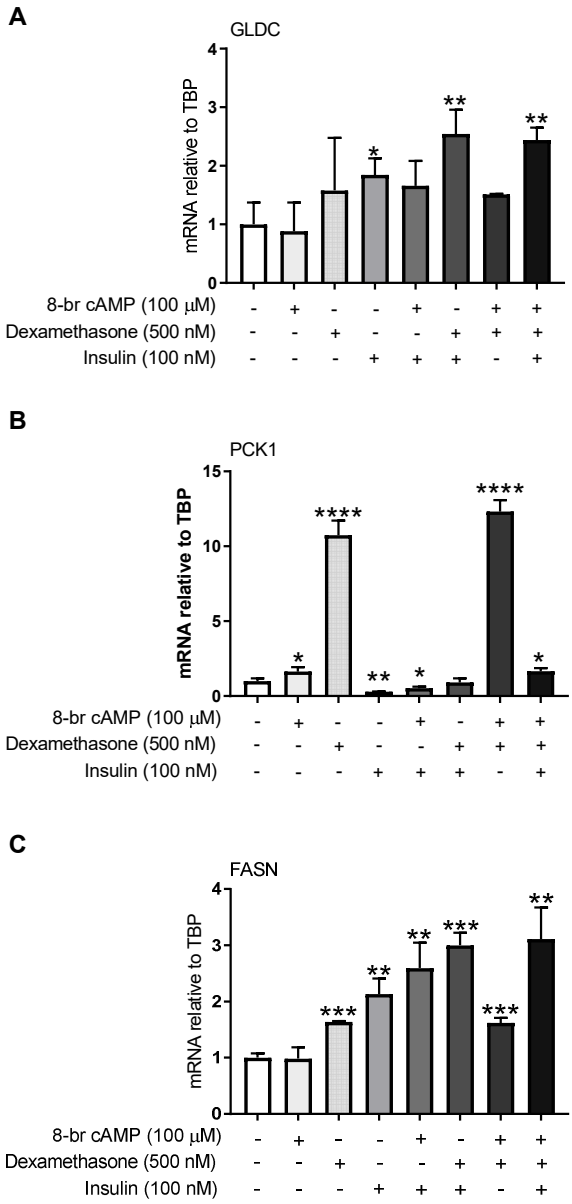

Figure 3.1: Hormonal regulation of GLDC mRNA

H4IIE cells were plated in DMEM complete media and incubated overnight. The next day the medium was replaced with DMEM devoid of serum containing either individually or a combination of 8-bromo cyclic AMP (100 uM), dexamethasone (500 nM) and insulin (100 nM) or vehicle. After 6h of incubation, cells were harvested for extraction of total RNA. The abundance of (A) GLDC mRNA, (B) PCK1 mRNA and, (C) Fasn mRNA in total RNA were measured relative to TBP (Tata binding protein) as described under "materials and methods". Values for cells incubated with vehicle alone were set at 1 and the other values were adjusted proportionately. Values are means $\pm$ S.D. of three experiments. Data were analyzed used one-way ANOVA followed by Tukey's post hoc test. Bars that share the same letter are not significantly different.

3.3.2 **Regulation of GLDC by insulin in a rat hepatoma cell line, H4IIE.**

Based on the previous hormone panel, we decided to initially focus on the response of GLDC gene expression to insulin. When H4IIE cells were treated with either vehicle or insulin (100 nM) for 8h, GLDC mRNA levels were strongly and significantly upregulated (9.6 fold, P=0.0012) (Figure 3.2A). Our positive controls, FASN, and PCK1 responded to insulin as expected. FASN was significantly upregulated with insulin treatment (2.23 fold, P=0.0223) (Figure 3.2B). PCK1 mRNA, on the other hand, was significantly suppressed with insulin treatment (6.66 fold, P=0.0036) (Figure 3.2C). We observed this effect of insulin on the GLDC protein level as well (Figure 3.2D). We observed that the GLDC protein band in the rat hepatoma cell line was migrating at a lower than expected molecular weight (between 70-100 kDa, instead of at around 110 kDa). According to NCBI, the rat GLDC protein is 98 kDa. To confirm whether the band we are detecting is indeed GLDC protein, we used siRNA against rat GLDC and treated the cells with either vehicle or insulin. siRNA-mediated knockdown of rat GLDC markedly reduced basal levels, as well as the insulin, stimulated GLDC protein levels in the serum-free DMEM media condition (Figure 3.2E). Together these results demonstrate that GLDC expression is stimulated by insulin in hepatoma cells. During the establishment of this experimental system, we noticed a striking effect of serum on GLDC protein levels, which is explored in the next section (Figure 3.3)

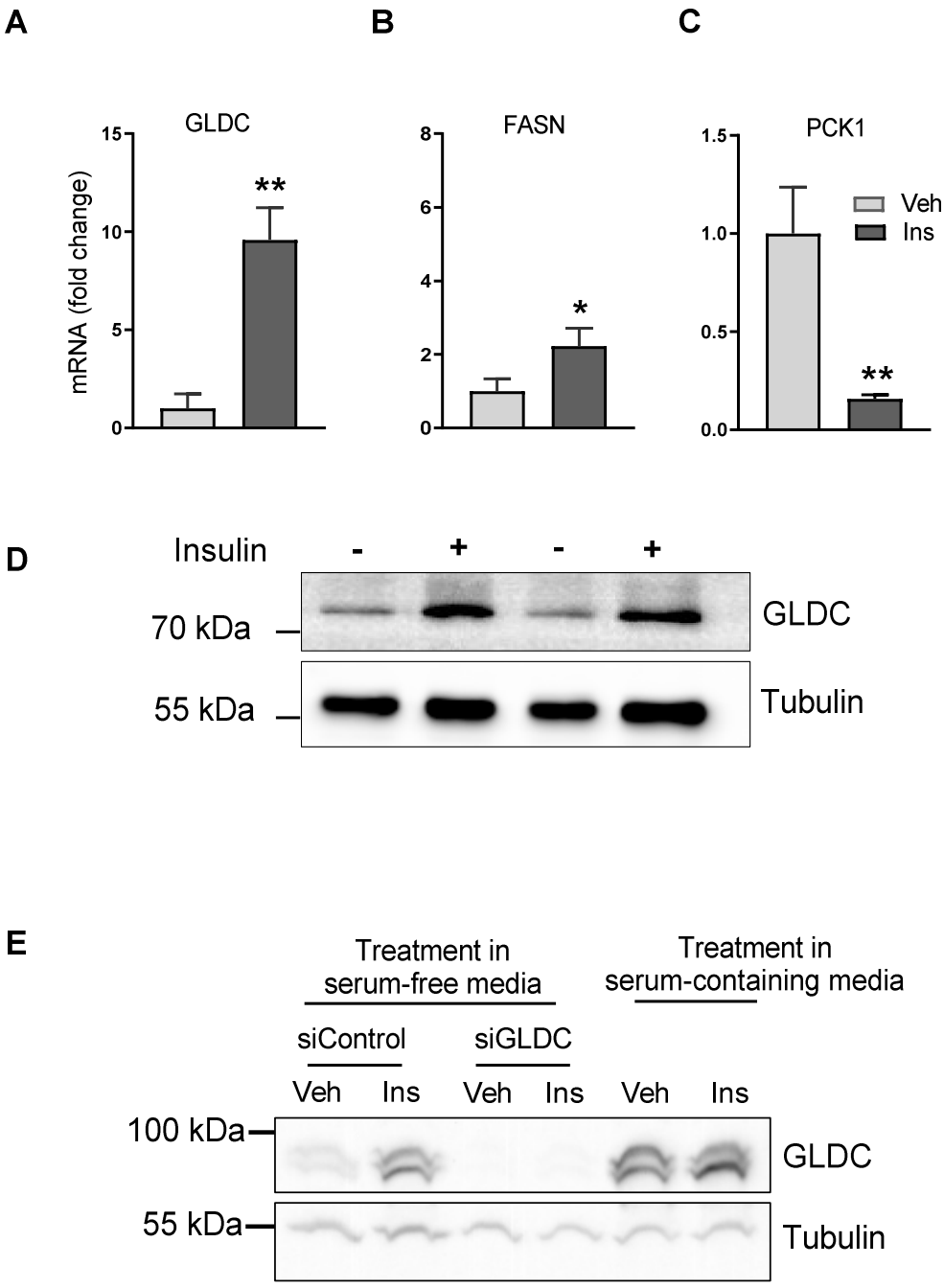

Figure 3.2: Regulation of GLDC by insulin in a rat hepatoma cell line, H4IIE
H4IIE cells were plated in DMEM complete media and incubated overnight. The next day the medium was replaced with DMEM devoid of serum containing insulin (100 nM) or vehicle. After 8h of incubation, cells were harvested for extraction of total RNA. The abundance of (A) GLDC mRNA, (B) FASN mRNA and, (C) PCK1 mRNA in total RNA were measured as described under "materials and methods". Values for cells incubated with vehicle were set at 1 and the other values were adjusted proportionately. Data were analyzed using $2^{-\Delta\Delta Ct}$ method and quantitated by RT-qPCR. TBP was used as a reference gene. Error bars mean $\pm$ s.d.; *P<0.05, **P<0.01. Results were confirmed with three independent experiments. (D) Western blot of

GLDC protein levels after insulin (100 nM) treatment for 24h in H4IIE cells. Tubulin was used as a loading control. (E) Verification of the GLDC protein in H4IIE cells. H4IIE cells were transfected with control siRNA or siRNA targeting GLDC as described under “materials and methods”. After transfection, cells were incubated in either complete DMEM media or serum-free DMEM containing insulin (100 nM) or vehicle for 24h. Cells were then harvested for preparing protein lysates and immunoblotted for the indicated proteins.

3.3.3 **Regulation of GLDC by glucose and fetal bovine serum.**

We tested the effect of serum on GLDC protein levels by withdrawing serum from H4IIE cell cultures for 3h, 6h, 12h or 24h. We observed a time-dependent decline in GLDC protein levels after serum withdrawal with a nearly total loss of expression by 24 hours (Figure 3.3A). This suggests that one or more serum components are necessary for maintaining basal GLDC expression. Given the stimulatory effect of insulin observed in (Figure 3.2A), one possibility is that the crucial serum component is insulin.

In addition to hormonal abnormalities type 2 diabetes is also characterized by hyperglycemia, raising the possibility that glucose itself could be a regulator of GLDC gene expression. To assess the effect of glucose on GLDC expression H4IIE cells were grown in DMEM media containing low (5.5 mM) or high (27.5 mM) glucose and treated with vehicle or insulin for 8h. While insulin had the expected stimulatory effect on GLDC gene expression, the concentration of glucose had no impact on either the basal or insulin-stimulated level of GLDC expression (Figure 3.3B). As a control, we also examined the expression of the PCK1 gene, which is known to be suppressed by glucose (119, 120). As shown in (Figure 3.3C), the responsiveness of PCK1 to insulin was enhanced by glucose. Together, these findings indicate insulin prevents the loss of GLDC expression induced by serum starvation, and this effect is not affected by glucose.

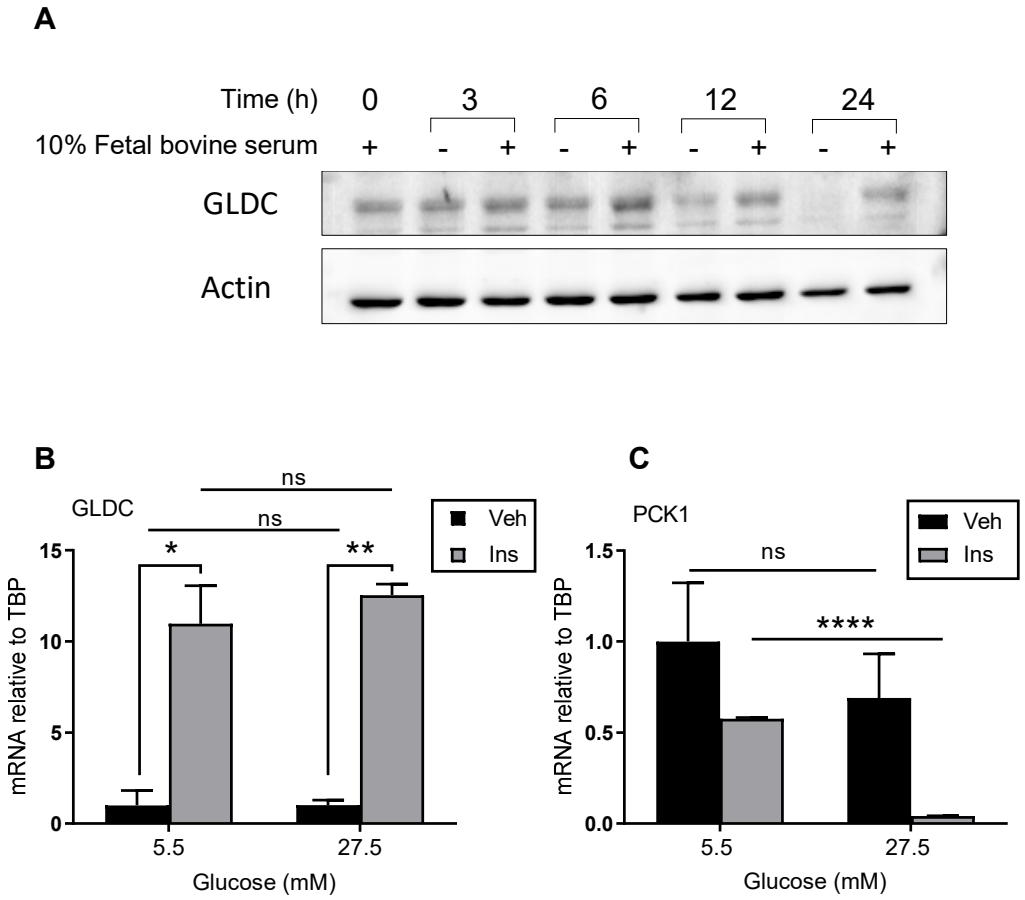

Figure 3.3: Regulation of GLDC by glucose and fetal bovine serum
(A) Western blot of GLDC protein levels in H4IIE cells at indicated time points grown in DMEM media either containing 10% fetal bovine serum or devoid of 10% fetal bovine serum. Actin was used as a loading control. (B, C) H4IIE cells were incubated overnight in either DMEM complete media containing 5.5 mM or 27.5 mM glucose. The next day medium was replaced with DMEM devoid of fetal bovine serum with either 5.5 or 27.5 millimolar glucose containing either vehicle or insulin (100 nM). After 8 hours of incubation, cells were harvested for total RNA extraction and analysis by RT-qPCR. The abundance of (A) GLDC mRNA and (B) PCK1 mRNA in total RNA was measured as described in "methods" section. Values for cells incubated with vehicle were set at 1 and the other values were adjusted proportionately. Data were analyzed using $2^{-\Delta\Delta Ct}$ method and quantitated by RT-qPCR. TBP was used as a reference gene. Error bars mean $\pm$ s.d.; *P<0.05, **P<0.01, ****P<0.0001.

3.3.4 **SREBP1c regulates GLDC transcription**

The strong effect of insulin on GLDC gene transcription prompted us to examine potential transcriptional regulatory pathways that might mediate this effect.

Because many hepatic expressed genes that respond to metabolic hormones are regulated by the FOX family of transcription factors, we sought to determine if GLDC was a target of the FOX proteins. Systematic bioinformatics analysis of the GLDC promoter identified two evolutionarily conserved putative binding sites for the transcription factor FoxA2 in the proximal promoter of the human GLDC gene (Figure 3.4 A). These sites were between - 602 and - 425 in the human GLDC promoter. To determine if these were indeed functional FoxA2 regulatory elements, we performed luciferase reporter assays with varying lengths of GLDC promoter (1.74 kb, 1 kb, and 600 bp) and overexpression of FoxA2. Figure 3.4 (B), is an example western blot of transient transfection of FoxA2 in either HepG2 or HEK293A cells and measuring Foxa2 and GLDC protein levels. GLDC protein levels were not altered with FoxA2 overexpression. We also measured GLDC promoter activity with the other members of the FoxA family (A1, A3) and FoxO1 but did not see a change in GLDC promoter activity or protein levels. We concluded that under all the conditions tested, GLDC was not regulated by the FOXO family of transcription factors.

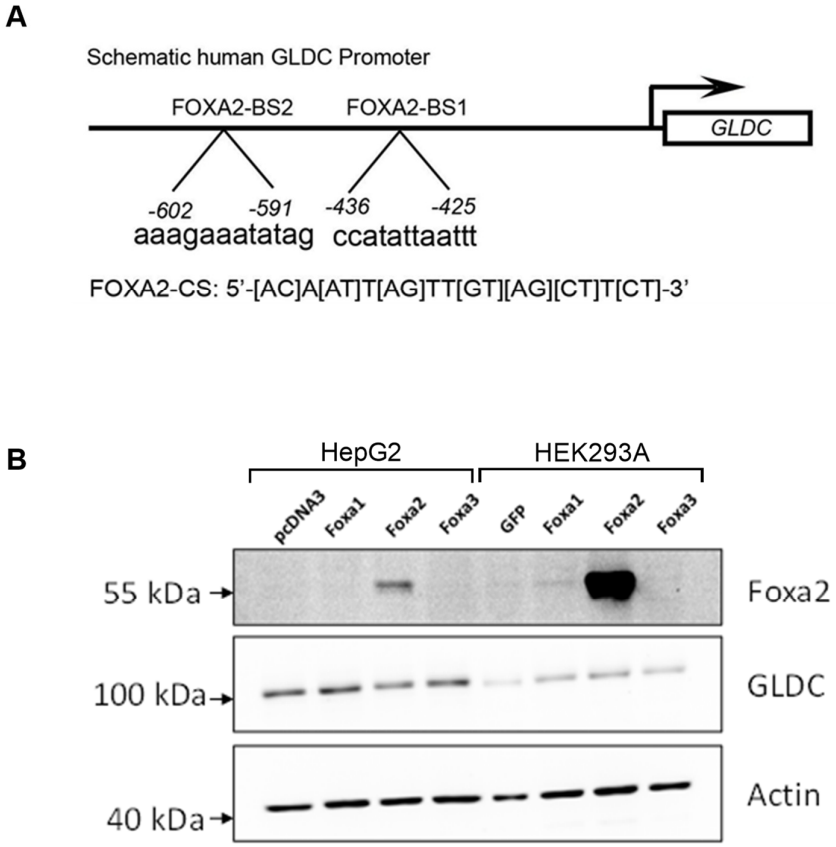

Figure 3.4: GLDC promoter contains putative binding sites for the transcription factor FoxA2

Schematic diagram of human GLDC promoter showing putative FoxA2 binding sites identified using PROMO. BS- binding sites; CS - consensus sequence. (B) Fox A1 A2 and A3 plasmids along with the control plasmid pcDNA3 were transfected in HepG2 and HEK293A cell lines and extracts were immunoblotted for the indicated proteins. Representative data are shown from three independent experiments.

Another transcription factor known to mediate insulin's effect on the transcription of a subset of hepatic genes, including FASN, is the transcription factor SREBP1c. To begin exploring the possibility that Srebp1c mediates the effect of insulin on GLDC transcriptional activity, we examined SREBP1c ChIP-Seq data from ENCODE/SYDH database from insulin-treated HepG2 cells (Figure 3.5 A). This global data set of functional Srebp1c binding sites identified a Srebp1c signal in the proximal promoter of the GLDC gene. The location of this SREBP binding signal was consistent with the location of evolutionarily conserved E-box and SRE motifs (identified using Matinspector), which are classified as putative Srebp1c binding sites.

To experimentally confirm that SREBP1c is a functional regulator of GLDC gene transcription, we directly examined its effect on GLDC promoter activity in a transient transfection reporter assay. Exogenous SREBP1c and GLDC promoter-luciferase reporter were transfected into the human hepatoma cell line Huh7. In this system, GLDC transcriptional activity was stimulated 4.9 fold (P=0.0019) by the introduction of exogenous SREBP1c (Figure 3.5 B). FASN, a well-characterized SREBP1c responsive gene included as a positive control, was stimulated nearly 9 fold by SREBP1c in this system(Figure 3.5 C). Similar results were obtained in HEK293T cells after ectopic overexpression of SREBP1c where transfection of SREBP1c stimulated GLDC transcriptional activity by 4.32 fold (P=0.0228) (Figure 3.5 E-G). We also examined the effect of ectopic SREBP1c on the expression of the endogenous GLDC gene in HEK293T cells. Also, in this case, SREBP1c significantly stimulated endogenous GLDC mRNA levels (2 fold, P=0.0024) (Figure 3.5 H). Together these data demonstrate that SREBP1c regulates GLDC transcription and although we were not able to confirm this experimentally (by using a dominant negative SREBP1c expression plasmid), they strongly suggest that the observed stimulation of GLDC transcription by insulin in hepatoma cells is mediated by an SREBP1c -dependant mechanism.

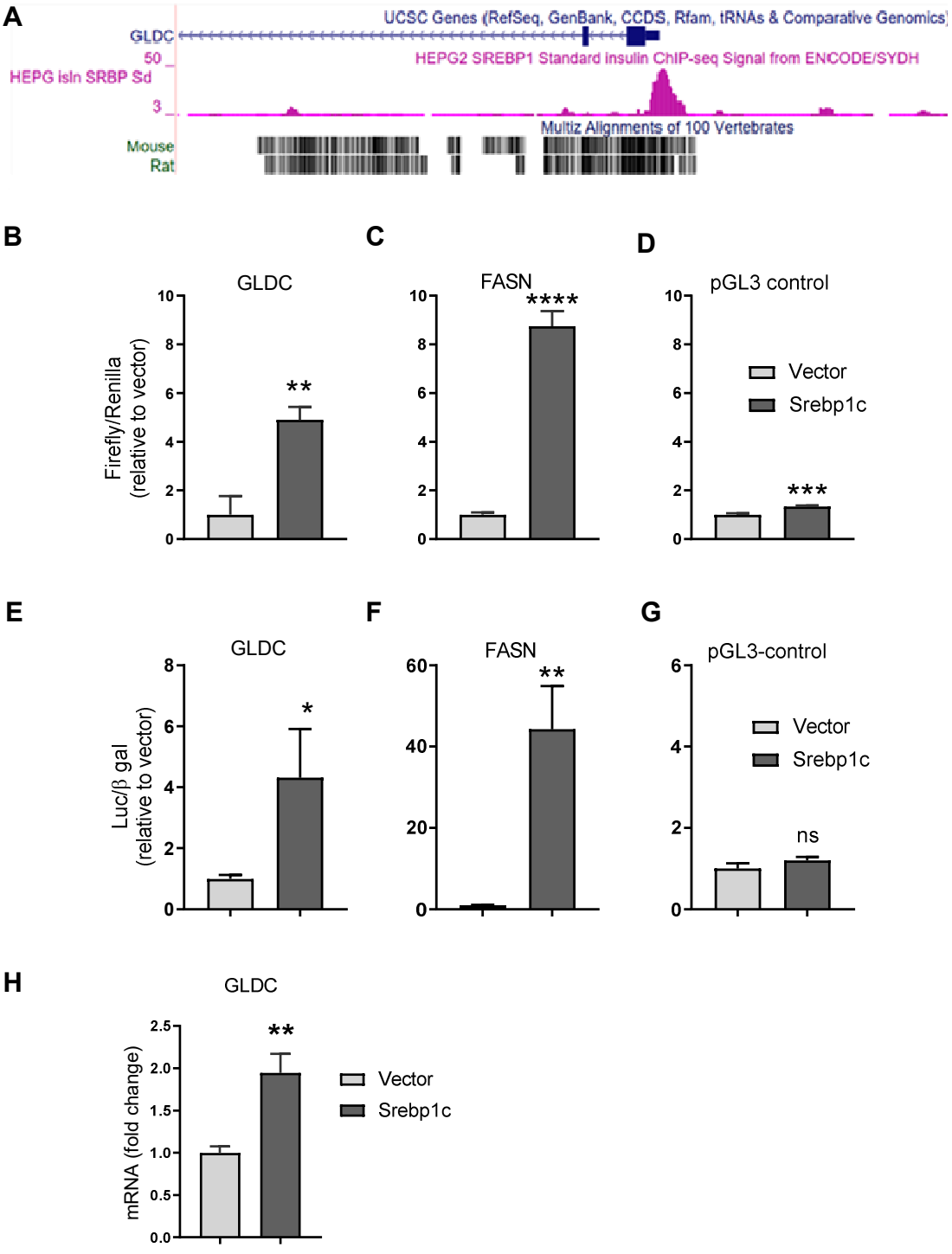

Figure 3.5: Regulation of GLDC by the insulin-responsive transcription factor, sterol regulatory element-binding protein 1c (SREBP1c)

(A) GLDC promoter contains putative Srebp1c binding sites. Genome-wide characterization of Srebp1c-binding sites by ChIP-Seq in human GLDC promoter (Reed, Charos et al. 2008) in insulin-treated HepG2 cells. The figure image was generated from ENCODE/SYDH. (B-D)

GLDC promoter activity is regulated by SREBP1c in Huh7 cells. (B) GLDC, (C) FASN, and (D) pGL3-control luciferase reporters co-transfected with pcDNA3.1-SREBP1c. Data are mean ± S.D., n=3, **P<0.01, ***P<0.001, ****P<0.0001. The cells were then harvested for the measurement of luciferase activities. Each column represents the mean ± S.D. of normalized (Firefly/Renilla) luciferase measurements relative to vehicle control. (E-G) GLDC promoter activity is regulated by SREBP1c in HEK293T cells. (E) GLDC, (F) FASN, and (G) pGL3-control luciferase reporters co-transfected with pcDNA3.1-SREBP1c. Data are mean ± S.D., n=3; *P<0.05, **P<0.01. The cells were then harvested for the measurement of luciferase activities. Each column represents the mean ± S.D. of normalized (luciferase units/beta galactosidase) luciferase measurements relative to vehicle control. (H) SREBP1c regulates endogenous GLDC mRNA levels. GLDC mRNA levels after transient transfection of pcDNA3.1-SREBP1c in HEK293T cells. Data are mean ± S.D., n=3; **P<0.01.

3.3.5 **Hormonal regulation of GLDC in rat primary hepatocytes**

The fact that GLDC levels are elevated by fasting suggests that GLDC gene expression might be regulated by glucagon. Since our H4IIE cells did not show a robust response to cAMP (Figure 3.1 A), we examined this possibility in rat primary hepatocytes, which are considered a gold standard for hepatic gene expression studies. To determine if GLDC was regulated by glucagon or its downstream signal, we treated primary hepatocytes with either vehicle, db-cAMP (100 μM) or glucagon (100 nM) for 12h. GLDC expression was stimulated 18.1 fold by db-cAMP ($P<0.0001$) and by 17.5 fold by glucagon ($P<0.0001$) (Figure 3.6 A). PCK1 and FASN served as controls and responded as expected to both db-cAMP and glucagon (Figure 3.6 B and C). The changes in GLDC mRNA levels were reflected in the levels of GLDC protein (Figure 3.6 D). Of the four genes that comprise the glycine cleavage system, only GLDC was responsive to db-cAMP treatment (Figure 3.6 E).

We also examined the effect of insulin on GLDC expression in primary hepatocytes. Similar to the H4IIE cells, although to a lesser degree, insulin stimulated GLDC transcription (1.7 fold, $P=0.019$) in primary hepatocytes (Figure 3.6 F). To confirm the insulin-responsiveness of the cells, we measured the response of FASN and PCK1 mRNA levels to insulin treatment. Insulin stimulated FASN mRNA Figure by 2.34 fold ($P=0.0002$) (Figure 3.6 F) and suppressed the gluconeogenic gene PCK1 by 5.68 fold ($P=0.0064$) (Figure 3.6 F). Together these data indicate that glucagon is a potent inducer of GLDC gene transcription, and suggest that insulin, while also a positive regulator of GLDC expression, may function to maintain basal levels of GLDC expression.

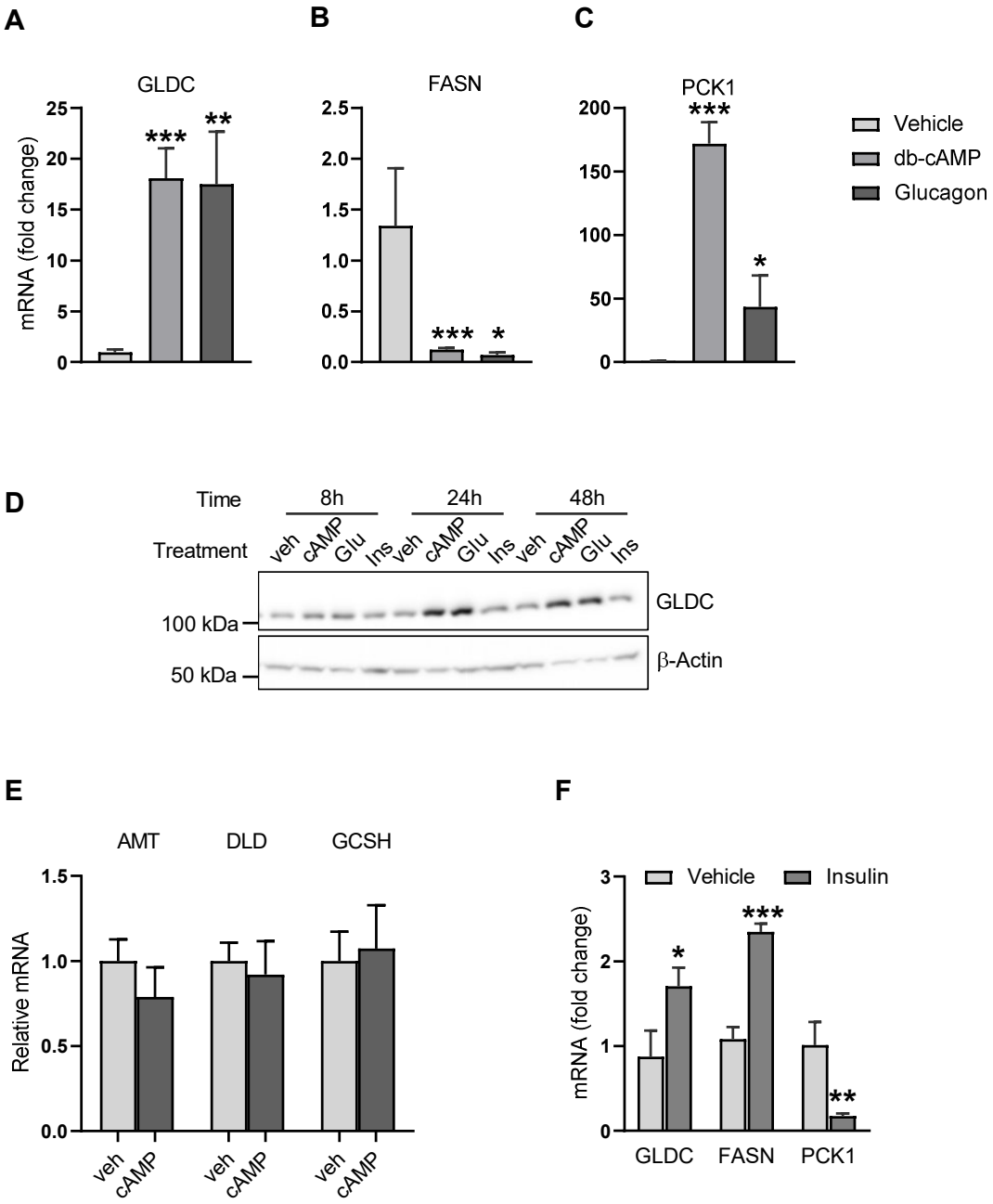

Figure 3.6: Regulation of GLDC by glucagon, cAMP and insulin in rat primary hepatocytes

48h after plating, rat primary hepatocytes were treated with either vehicle, db-cAMP (100 µM) or glucagon (100 nM). After 12 hours of incubation, cells were harvested for total RNA extraction and analysis by RT-qPCR. The abundance of (A) GLDC mRNA, (B) FASN mRNA and (C) PCK1 mRNA in total RNA was analyzed by $2^{-\Delta\Delta Ct}$ method and quantitated by RT-qPCR. PPIB was used as a reference gene. Error bars, mean ± S.D.; *P<0.05, **P<0.01, ***P<0.001. The results were confirmed with at least 3 independent rat hepatocyte preparations. (D) 48h after plating, rat primary hepatocytes were treated with either vehicle, db-cAMP (100 µM), glucagon (100 nM) or insulin (100 nM) for 8, 24 and 48h and GLDC protein was analyzed

by western blot. Actin was used as a loading control. (E) The abundance of AMT, DLD, and GCSH, the other components of the glycine cleavage system, was measured in hepatocytes treated with either vehicle or db-cAMP (100 μM). After 12 hours of incubation, the abundance of AMT, DLD, and GCSH mRNA in total RNA was analyzed by $2^{-\Delta\Delta Ct}$ method and quantitated by RT-qPCR. PPIB was used as a reference gene. Error bars, mean $\pm$ S.D.; *P<0.05, **P<0.01, ***P<0.001. The results were confirmed with at least 3 independent rat hepatocyte preparations. (F) 48h after plating, rat primary hepatocytes were treated with either vehicle or insulin (100 nM) for 12h, and GLDC, FASN, and PCK1 mRNA abundance was analyzed by $2^{-\Delta\Delta Ct}$ method and quantitated by RT- qPCR. PPIB was used as a reference gene. Error bars, mean $\pm$ S.D.; *P<0.05, **P<0.01, ***P<0.001. The results were confirmed with 3 independent rat hepatocyte preparations.

3.3.6 **CREB1 and ATF1 mediate cAMP-dependent activation of GLDC**

The glucagon-cAMP signaling pathway leads to the activation of CREB family (CREB1, ATF1, and CREM) transcription factors, which are known to mediate transcriptional effects of glucagon on the expression of many fasting-induced genes. In ENCODE database for CREB ChIP-seq, there were some putative binding sites for CREB transcription factors in the GLDC promoter. To determine if CREB proteins also mediate the effects of glucagon on GLDC expression, we performed siRNA mediated CREB1 and ATF1 knockdown in primary hepatocytes (Figure 3.7). Knockdown of CREB1 (isoform A and B) and of ATF1 reduced the ability of db-cAMP to stimulate GLDC transcription (Figure 3.7 A). The effect of CREB1 knockdown was seen at GLDC protein levels as well (Figure 3.7 B). CREB1 and ATF1 are known to either homodimerize or heterodimerize to regulate target gene expression. When we performed a double knockdown of CREB1 and ATF1, we did not see a stronger reduction of GLDC mRNA (Figure 3.7 A) than either individual knockdown. This suggests that the configuration of CREB transcription factors with regard to GLDC expression is a CREB1 and ATF1 heterodimer.

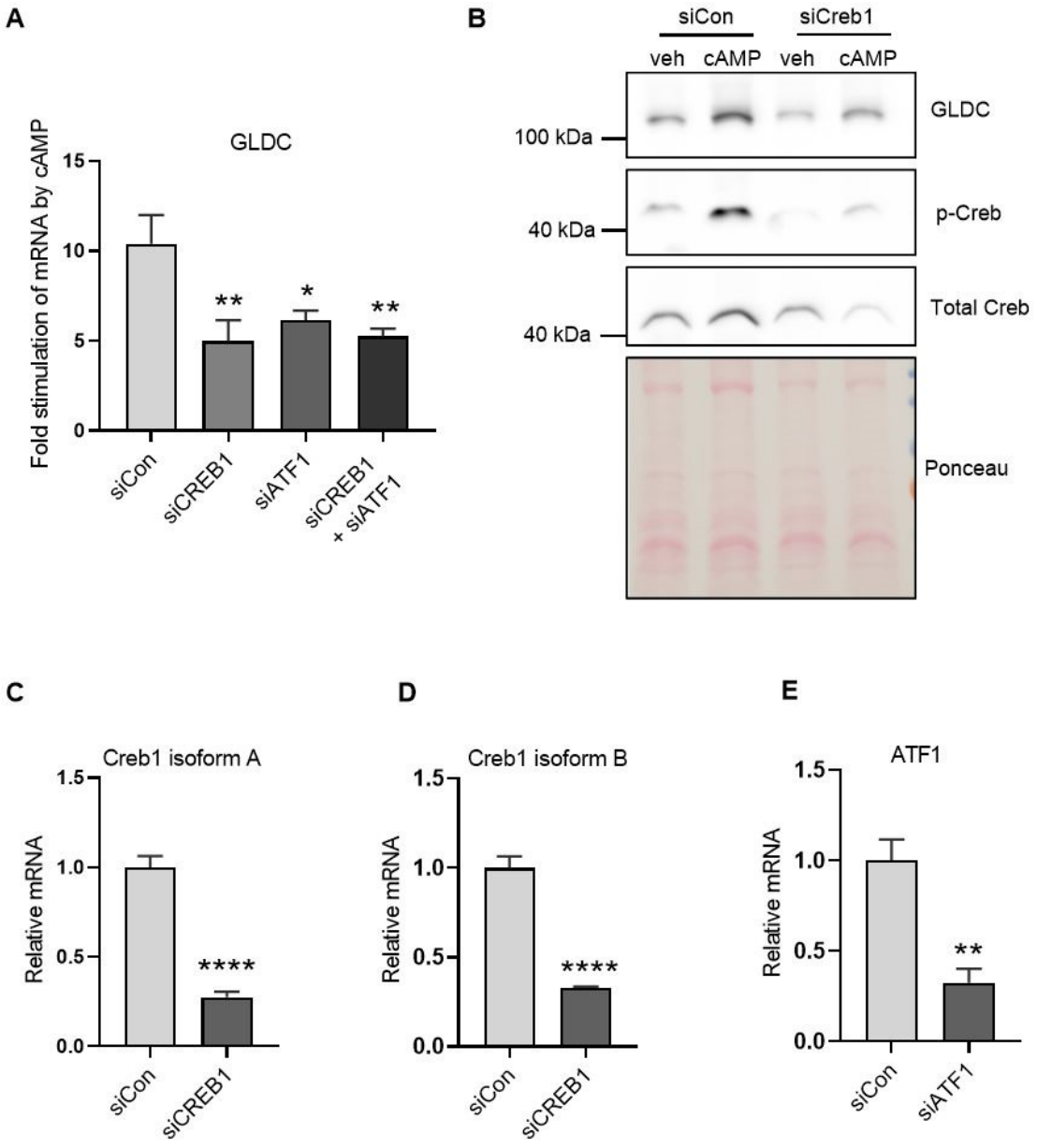

Figure 3.7: CREB1 and ATF1 mediate cAMP-dependent activation of GLDC
(A) GLDC mRNA abundance with cAMP stimulation in rat primary hepatocytes transfected with siRNA against CREB1 and/or ATF1. (B) Transfection of siRNA against scrambled control and Creb1 in rat primary hepatocytes for 48h and then treated with either vehicle (veh) or cAMP for 20h. GLDC, p-Creb and total Creb protein levels quantified by western blotting normalized to Ponceau staining of the membrane. (C) Creb1 isoform A mRNA, (D) Creb1 isoform B mRNA, and (E) ATF1 mRNA knockdown in rat primary hepatocytes was confirmed by RT-qPCR. Error bars, mean $\pm$ s.d.; *P<0.05, **P<0.01, ****P<0.0001. The results were confirmed with at least 3 independent rat hepatocyte preparations.

3.4 Discussion

In the previous chapter, we showed that hepatic GLDC expression was stimulated by fasting and in two mouse models of diabetes, and proposed that this effect contributed to the reduced glycine observed in those states. The objective of the work described in this chapter was to identify the specific signaling pathways and transcriptional mechanisms responsible for the regulation of GLDC expression in these states. Type 2 diabetes is accompanied by high levels of circulating insulin, glucagon, and glucocorticoids depending on the stage of the disease and its etiology. Given that these hormones can all stimulate GLDC expression, it seems likely that the reduced levels of glycine seen in both early and late-stage diabetes and in other metabolic disease syndromes such as obesity and NAFLD are due, at least in part, to elevated expression of GLDC mediated by one or more of these hormones.

We found that GLDC was positively regulated by the metabolic hormones glucagon, insulin, and cortisol. We further demonstrated that glucagon modulates GLDC expression via the PKA-mediated activation of CREB1/ATF1 transcription factors. We also presented data strongly suggesting that SREBP1c mediates the insulin effect on GLDC transcription. Given that we did not observe any effect of glucose levels on GLDC expression, we conclude that the primary regulatory pathways acting on GLDC, in this context, are hormonal. Of particular interest, in H4IIe cells, insulin was the dominant hormone for the regulation of PCK1 gene. In these cells, PCK1 was unresponsive to glucagon-mediated stimulation and at the same time hypersensitive to insulin-mediated suppression of gene expression. In rat primary hepatocytes, which more closely resemble liver cells *in vivo* than H4IIE hepatoma cells, glucagon was the dominant hormone. This is consistent with a previous report that shows the flux of the glycine cleavage system is stimulated with glucagon (121).

Our observation that glucagon only stimulates GLDC and not the other components of the glycine cleavage system is consistent with the idea that GLDC is the rate-limiting component of the glycine cleavage system and the key enzymatic pathway that controls blood glycine levels (122).

What could be the physiological significance of GLDC regulation by glucagon? Glycine catabolism by the glycine cleavage system is an essential metabolic process that feeds into the one-carbon pathway system**.** The flux through the glycine cleavage system has been shown to be stimulated by glucagon (121) and high-protein meal (123). We have shown that fasting, glucagon, and cAMP stimulate GLDC gene expression. Glucagon is known to stimulate the flux through oxidative phosphorylation (124-126). We speculate that the physiologic significance of glucagon regulation of GLDC is to meet cellular energy requirements in the form of ATP through mitochondrial respiration and stimulation of gluconeogenesis during the fasted state. One of the products of glycine catabolism is the production of NADH (70) which can be utilized towards glucose production through two routes. This mitochondrial NADH can donate electrons to the electron transport chain to generate ATP (127) which would allow ATP-dependent processes such as pyruvate carboxylation to proceed at a faster rate aiding gluconeogenesis (128). Mitochondrial NADH can be shuttled to the cytosol by the malate-oxaloacetate shuttle, thereby contributing to the cytosolic NADH pool required for gluconeogenesis (129). The speculation of the link between GLDC and gluconeogenesis is further supported by the hepatic and renal distribution of GLDC (69, 98) which are main sites of glucose production. Even within the metabolic zonation of the liver, GLDC is expressed in the periportal region which is the site for gluconeogenesis (130).

A curious aspect of our findings is that both glucagon and insulin stimulate GLDC. These are generally considered counter-regulatory hormones. For example, PCK1 is stimulated by glucagon and suppressed by insulin. The only other gene that we know of that is similarly

regulated by insulin and glucagon is FGF21 (regulator of carbohydrate and lipid metabolism). FGF21 expression is cooperatively stimulated by both insulin and glucagon (neither hormone had an effect on its own (131). GLDC regulation differs from FGF21 in that it was stimulated by the individual hormones, and that they did not appear to function in a cooperative manner.

Are there any physiological circumstances where glucagon and insulin might act together to enhance GLDC expression?- The only circumstance where the production of both hormones by the pancreas occurs simultaneously is after a pure protein meal. In these circumstances it may be beneficial to have an elevated GLDC expression and glycine degradation. In fact, it is known that glycine levels are reduced by a high protein diet, consistent with this idea that GLDC expression is elevated under these circumstances. A high-protein diet in rats is associated with reduced plasma glycine levels as compared to rats fed a low or normal protein diet (132). There is an increased hepatic uptake of glycine after rats are fed a high-protein diet (133). Even with the increased uptake, liver tissue glycine levels are reduced as seen in a study with rats adapted to a 5, 13, and 50% casein diet for 21 days, and liver glycine levels were 4830, 2080, and 580 nmoles/gm of liver tissue, respectively (134).

In addition, it has been shown that the flux through the hepatic glycine cleavage system is rapidly stimulated with a high-protein meal (123). In rats adapted to a high protein vs a low protein diet for 24 days, the diurnal glycine levels increased during the daytime on the high-protein diet. But the glycine levels were overall 3-4 fold lower at all times on a high-protein as compared to a low-protein diet (135).

An interesting finding was the requirement of serum in growth media to maintain GLDC expression levels. In the absence of serum, insulin alone was able to maintain GLDC expression levels in H4IIE cells. This observation suggests that insulin acts mainly to ensure a basal level of expression of GLDC expression, for example after a meal when glucagon is absent but when it would still be beneficial to have a functional glycine cleavage system.

The involvement of glucocorticoids in nutritional regulation of gene expression is somewhat complicated. Cortisol promotes protein catabolism (136), enhances gluconeogenesis (111), and in chronic excess (such as in Cushing's disease) causes insulin resistance (137). Our data revealed that insulin and dexamethasone had an additive effect in the stimulation of GLDC mRNA levels although we did not study the glucocorticoid regulation of GLDC in detail.

We tried using an SREBP1c dominant negative expression plasmid as well as siRNA against SREBP1c to reduce SREBP1c expression in H4IIE cells, treat the cells with insulin and meaure GLDC and FASN mRNA and protein levels. We were unable to see any effect of reducing SREBP1c expression on FASN or GLDC expression. Although we were not able to experimentally prove that the regulation of GLDC by insulin occurred through an SREBP1c pathway, we did clearly demonstrate that SREBP1c can stimulate GLDC gene expression. Given the well-characterized role of SREBP1c in insulin-mediated regulation of various hepatic genes such as FASN (138), it seems likely that stimulation of GLDC expression by insulin is also mediated by SREBP1c.

CREB1 and ATF1, well-characterized transcription factors, are targets of the cAMP-inducible PKA-mediated phosphorylation required for transcription activation. CREB1 and ATF1 are known to either homodimerize or heterodimerize to regulate target genes (139). We found that the configuration of CREB1 and ATF-1 heterodimer might be regulating GLDC transcription. This would be similar to glucagon-PKA-CREB regulation of other metabolic genes such as PCK1 and G6PC (140-143). Our observations that GLDC is upregulated by fasting and is positively regulated by glucagon is consistent with human studies on the metabolic effects of altering glucagon levels. A study by Boden et al. (1984) performed in 6 healthy normal-weight human subjects demonstrated the effects of selective glucagon deficiency (intravenous infusion of somatostatin + insulin) and excess (intravenous infusion of somatostatin + insulin + glucagon) on 21 plasma amino acids. From that panel, one of the largest changes was observed in glycine levels which increased (+24%) with glucagon deficiency and decreased (-20%) with glucagon excess (144). One of the frequently observed characteristics of type 2 diabetes is hyperglucagonemia (145, 146) and it is possible that reduced levels of glycine seen in diabetes and related disorders are due specifically to glucagon-mediated stimulation of GLDC gene expression. The role that this elevation of GLDC gene expression and accompanying reduction in glycine levels play in the development of diabetes requires further investigation.

CHAPTER 4 - IMPACT OF ALTERED GLDC GENE EXPRESSION ON CELLULAR PHYSIOLOGY

4.1 Introduction

The glycine cleavage system, and in particular the rate-limiting enzyme GLDC, are major determinants of glycine levels in circulation. This is illustrated most clearly by the effect of inherited inactivating mutations in GLDC, which cause extreme elevation of blood glycine levels and a condition referred to as non-ketotic hyperglycemia which is characterized by neurological and developmental disorders (77, 80, 81, 84). Similar effects are seen in animal models. As shown in Figure 4.1 B, loss of GLDC expression and its enzymatic activity in mice (using a gene-trap methodology) showed a significant increase in mean plasma glycine levels: 889 μM compared to 239 μM in wild-type mice (77). The key role of GLDC in determining glycine levels is clearly seen in cell culture experiments where overexpression of GLDC led to a reduction in glycine levels in both HLF and NIH 3T3 cells, and retroviral knockdown of GLDC in A549 cells led to an increase in glycine levels as measured by LC-MS based metabolic profiling (Figure 4.1 A) (86).

An aberrant overexpression of GLDC is observed in various types of cancers, barring a single report of GLDC promoter hypermethylation and silencing in gastric cancer (91). GLDC is overexpressed in the tumor-initiating cells in non-small cell lung cancer (86), breast neoplasms (88), glioblastoma multiforme (87), and prostate adenocarcinoma (147). Whether there exists a relationship between changes in GLDC expression observed in different cancer types and in our results is unclear. But there is a report that shows a decrease in plasma glycine levels in lung cancer patients (148).

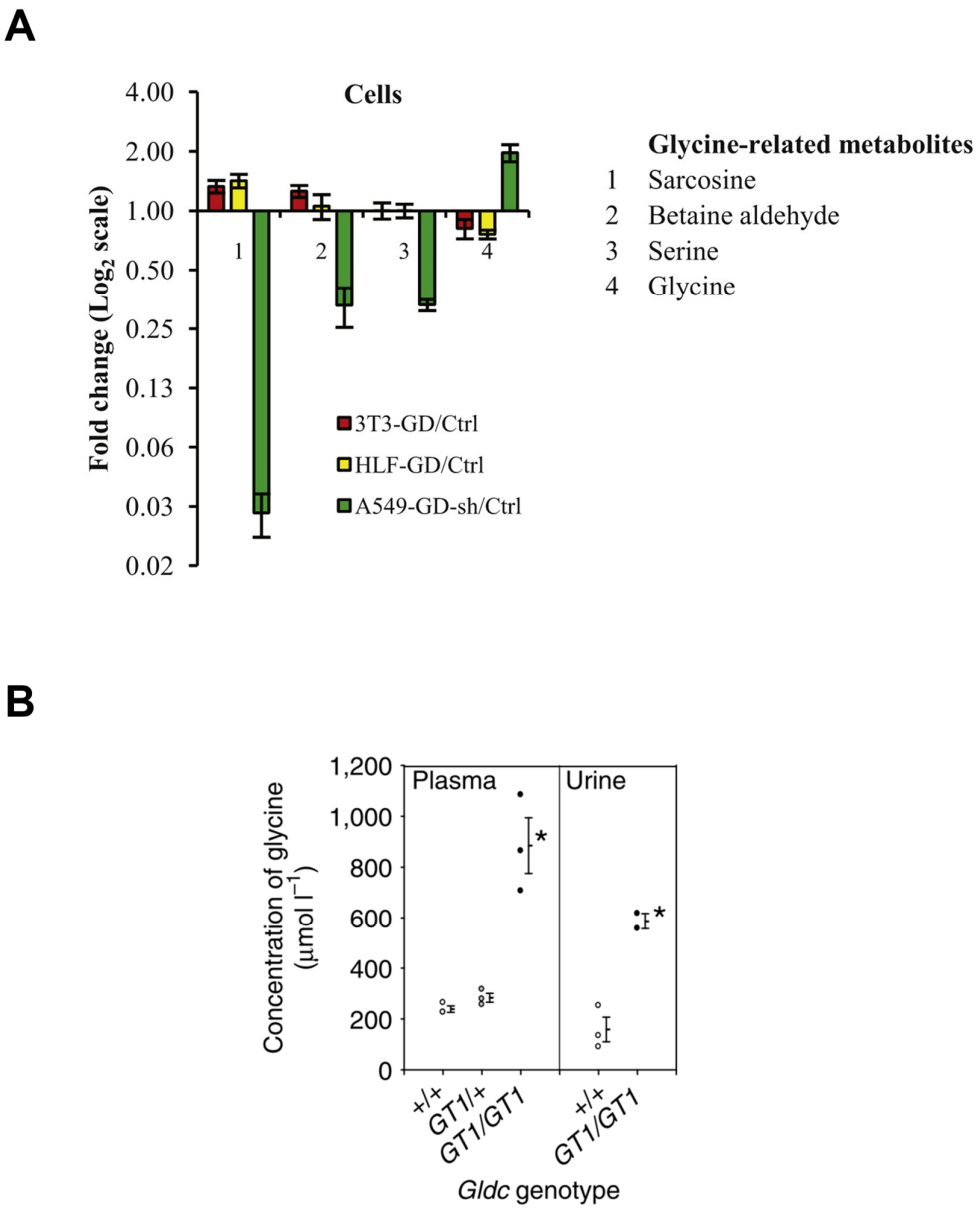

Figure 4.1: Effect of altering GLDC on glycine levels.
(A) Overexpression of GLDC in 3T3 and HLF cells, knockdown of GLDC in A549 cells and relative fold change in glycine and glycine related metabolites (86). (B) Elevated glycine concentrations in plasma and urine of GT1/+ and GT1/GT1 (GLDC gene trap models) mice as compared to GLDC+/+ mice (77).

As reviewed in Chapter 1, glycine and its metabolic derivatives are deeply integrated into a variety of critical metabolic pathways. Of particular interest is its role in the biosynthesis of glutathione, the principal cellular antioxidant. Glycine is most clearly linked to glutathione metabolism by the fact that it is one of the three amino acids that compose the glutathione molecule. It is also possible that the production of 1C groups generated during glycine catabolism also indirectly contribute to glutathione production. The methylene tetrahydrofolate generated by GLDC and the glycine cleavage system can be shuttled through the folate-methionine-transsulfuration pathway and end up contributing towards glutathione biosynthesis by providing carbons for the synthesis of the cysteine needed for the first step in glutathione synthesis (Figure 4.2). Thus it is possible that altering the levels of GLDC could affect glutathione production by either reducing the availability of glycine or by increasing the availability of cysteine. Considering that oxidative stress is one of the hallmarks of type 2 diabetes, alteration in cellular glutathione levels could modify the risk of developing diabetes or change its rate of progression. Another potential physiological outcome of altered GLDC expression could be related to another product of glycine degradation by GLDC: NADH. As the mitochondrial glycine cleavage reaction takes place in the mitochondrial matrix in close proximity to the electron transport chain, NADH produced from glycine degradation can act as an electron donor for oxidative phosphorylation and thereby could alter mitochondrial respiration and energy output.

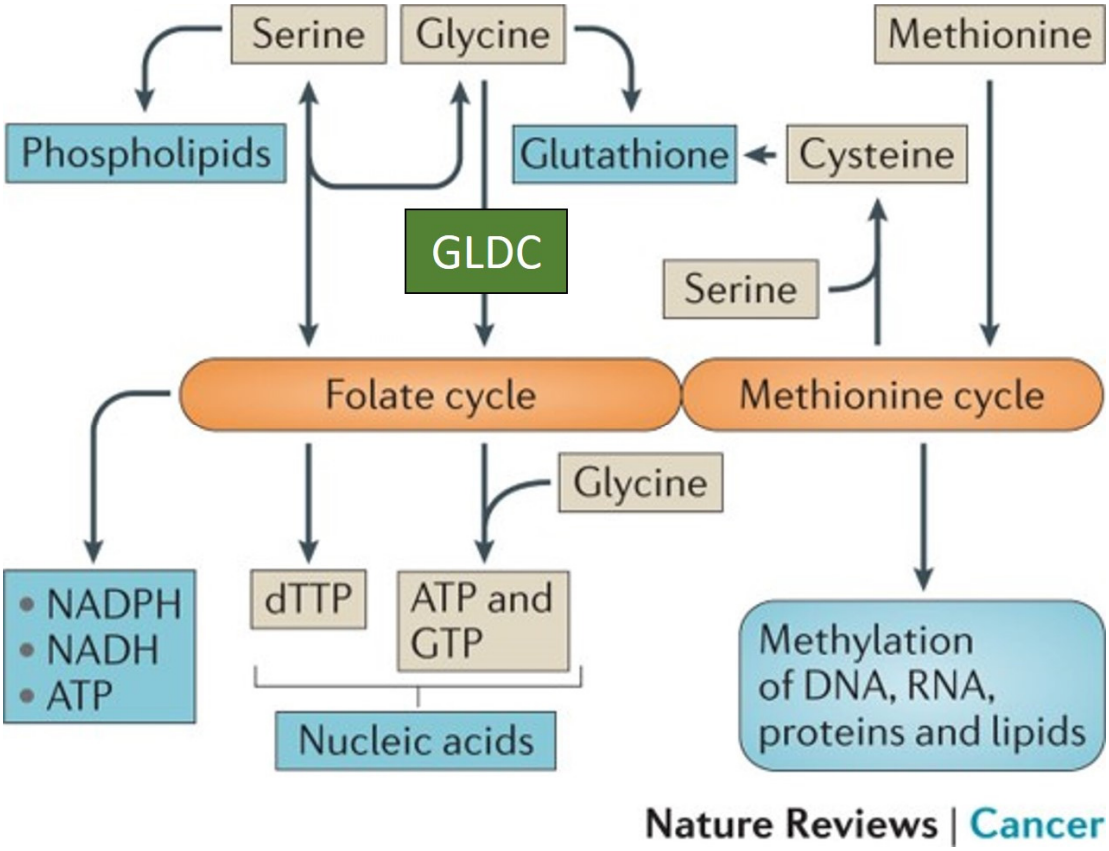

Figure 4.2: Role of serine and glycine in one-carbon metabolism.
Adapted from (64)

4.2 Materials and methods

4.2.1 **Plasmid generation and cell line establishment**

The construction of the luciferase reporters for GLDC promoter is described in an article elsewhere (Chen G et al. (2018)).

For stable shRNA-mediated knockdown of GLDC, the pLKO.puro lentiviral small-hairpin RNA vectors against human GLDC (TRCN0000303371) was obtained from Sigma. The lentiviral packaging strategy has been explained in a previous publication (149). For stable knockdown of GLDC, HepG2 cells were infected with pLKO.puro-GLDC and selected with 2 µg puromycin/ml. The puromycin-resistant cells were validated for altered GLDC expression by western blotting.

For stable Crispr/Cas9-mediated GLDC knockout, the construction of the targeting vector against human GLDC, the sgRNA sequence was determined based on the strategy explained in a previous article (150). The designed sgRNA sequence GGGTCTTTTCAAACGGATGT was annealed and cloned into pSpCas9(BB)-2A-Puro (Addgene) and used for generation of the targeting vector that was transfected into HEK293A cells using Lipofectamine 2000 (Invitrogen). The puromycin-resistant cell clones were selected on 1 µg puromycin/ml and screened for loss of GLDC protein expression by western blotting.

For stable GLDC knockdown or knockout cells, individual colonies were isolated from a heterogeneous pool for experiments. Cells were serially diluted and plated for distinct isolated colony formation. These colonies were then picked under a light microscope placed inside a laminar air flow cabinet. Each coloniy was trypsinized and plated in a well of a 48 well tissue culture plate and incubated until cells reached confluence of 75-80%. Cells were subsequently moved into a 12 well plate and then into a 6 well plate. Knockdown or knockout of GLDC was confirmed by western blotting.

4.2.2 **Western blot analysis from cultured cells**

Cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS), scraped off the plates, and lysed in RIPA lysis and extraction buffer (Thermo Scientific) with protease inhibitors followed by sonication for 30 seconds. Protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Scientific). Cell lysates (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk in PBS, pH 7.4, containing 0.1% Tween-20 (PBST).

The protein bands were visualized with enhanced chemiluminescence reagents (Perkin Elmer). Primary antibodies used GLDC (Sigma), AMT (Sigma), DLD (Sigma), phospho-CREB (CST), total CREB (CST), GAPDH (Santa Cruz), and Actin (Sigma). The signal was visualized using Azure c600 imaging system (Azure Biosystems). Ponceau staining of the membrane was used in some cases for normalizing the protein band intensity.

4.2.3 **Total glutathione measurement with an enzymatic recycling method**

Total glutathione was measured as described in (151). Briefly, HepG2 and HEK293A cells were cultured for 48h in GMEM media supplemented with 10% FBS, 0.4M serine and 0.4M glycine, 2 mM glutamine, penicillin, and streptomycin. Cells were washed twice with ice-cold, Ca2+-/Mg2+- free PBS. Cells were harvested with a cell scraper and placed in 1ml ice-cold PBS. Cells were pelleted by centrifugation at 1000g for 5 min at 4°C. The pellet was washed with ice-cold PBS twice. The pellet was resuspended in ice-cold extraction buffer (0.1% Triton-X and 0.6% sulfosalicylic acid in KPE (0.1M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5)) and cells were homogenized with rapid freeze-thaw in liquid nitrogen, thrice. The suspension was vortexed for 15 sec after each freeze-thaw.

The cells were centrifuged at 3000g for 4 min at 4°C, and the extract was used for GSH assay. For the assay, all solutions were prepared in 0.1 M KPE buffer, pH 7.5. The assay was

performed in a 96-well microtiter plate. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione reductase (GR) solutions were freshly prepared, mixed together, and added at 120 µl to each well. After allowing 30 sec for GSSG to be converted to GSH, 60 µl of β-NADPH was added to each well. The absorbance was read at 412 nm every 30 sec for 2 min (5 readings in total from 0-120 sec) and was repeated twice for each sample. The rate of 2-nitro-5-thiobenzoic acid formation (change in absorbance/min) was calculated. Total GSH concentration in the samples was determined by using linear regression to calculate the values obtained from the standard curve. Total GSH was normalized to the amount of protein in a separate set of samples plated, processed, and harvested at the same time.

4.2.4 **Total glutathione measurement using a luminescence-based system**

Total glutathione was measured using the GSH/GSSG-glo kit (Promega). Briefly, cells were plated at 10000 cells/well in a 96-well white wall, clear bottom plate. Cells were cultured for 48h in GMEM media supplemented with 10% FBS, 0.4M serine and 0.4M glycine, 2 mM glutamine, penicillin, and streptomycin. Cells were washed once with ice-cold, Ca2+-/Mg2+-free PBS. Total glutathione lysis reagent (Luciferin-NT, passive lysis buffer, water) 50 µl was added to the wells containing no cells (no-cell controls), cells, and to the standard curve wells. The plate was placed in a plate shaker for 5 min at room temperature. Luciferin generation reagent (100 mM DTT, glutathione-S-transferase, glutathione reaction buffer) 50 µl was added to the wells and the plate mixed briefly and incubated at room temperature for 30 min. Luciferin detection reagent 100 µl was added to the wells, mixed, and incubated for 15 min. Luminescence was measured on a CLARIOStar plate reader.

4.2.5 **ROS measurement using CM-H_2DCFDA**

General cellular ROS was measured using CM-H_2DCFDA dye (Invitrogen). Cells were plated in black wall, clear bottom 96 well plates at 15,000 cells/well in DMEM complete media and cultured for 24h. The oxidative stress inducer, tert-butyl hydrogen peroxide (tBHP), was

added on the cells in DMEM complete media at concentrations indicated in the figures for 4h at 37°C at 5% CO2. Cells were washed twice with $Ca2^{+}/Mg2^{+}$- free PBS. CM-H_2DCFDA was added on the cells at a concentration of 10 µM for 30 min at 37°C at 5% CO2 in DMEM Fluorobrite media (Invitrogen). Cells were washed twice with $Ca2^{+}/Mg2^{+}$- free PBS. The plate was read in a fluorescence plate reader set at Ex/Em of ~492-495/517-527 nm. The fluorescence intensity was normalized to the total protein from the same wells as measured by the Bradford method.

4.2.6 **Measurement of basal oxygen consumption rate**

Oxygen consumption rate was measured in intact HepG2 scrambled control and ShGLDC clones #1 and #3 cells on an XFe^{24} seahorse bioanalyzer. Before plating the cells the bioanalyzer plate was coated with gelatin (25 µl/well) and incubated at 37°C for 30 min. Excess gelatin was removed and the plate air-dried in the hood. 30000 cells per well were plated a night before. The basal oxygen consumption rate measurements were performed according to the manufacturer's instructions. After the measurement was complete, cells were lysed with RIPA (25 µl/well) buffer and equal volumes were loaded on an SDS-PAGE gel. The Western blot was probed for GAPDH and the band intensity was used for normalization of the oxygen consumption rate values.

4.2.7 **Cell proliferation**

HepG2 scrambled control and one clone each isolated from the two shGLDC guide targeting vectors (3371 and 6601) were plated on 6-cm plates and grown for 4 days in complete growth media. The seeding density was 25,000 cells/well for each cell type. Cells were washed with PBS and collected in trypsin. The cells, media and the PBS wash were mixed together and spun down at 1,000 RPM for 3 minutes at RT in a 15 ml Falcon tube. The pelleted cells were then resuspended in media and trypan blue staining (Invitrogen) was used to distinguish

between the dead and living cells. The counting was performed using a hemocytometer under a light microscope.

4.3 Results

4.3.1 **Establishment of mammalian cell lines with reduced or deleted GLDC.**

To understand the impact of altering GLDC expression on cellular physiology, several stable cell lines with either deleted of reduced expression of GLDC were generated. For GLDC knockdown in HepG2 cells, we used two targeting vectors against GLDC as described in the methods section of this chapter. The efficacy of GLDC knockdown using the heterogeneous cell pools from the two targeting vectors showed a more than 95% reduction in GLDC protein levels as compared to scrambled control. Measurement of protein levels of GLDC, DLD, and AMT, components of the glycine cleavage system showed that knockdown is specific to GLDC with both of the shGLDC targeting vectors (figure 4.3 A). Individual cell colonies were isolated by selection in 2 µg/ml puromycin from the heterogeneous cell pools and validated for GLDC knockdown by immunoblotting. The targeting vector 3371 had better efficiency of GLDC knockdown than targeting vector 6601 (figure 4.3 B). The individual clones generated from this targeting vector were used in our experiments. We also generated a Crispr/Cas9-mediated GLDC stable knockouts in HEK293A cells as another cell model system to study the effect of altering GLDC on cellular function. The methodology of Crispr/Cas9 sgRNA design and generation of the targeting vector is described in the methods section. Puromycin resistant heterogenous pool and individual cell colonies were screened for loss of GLDC by Western blot analysis. Most of the clones showed a complete knockout of GLDC expression (figure 4.3 C). The HEK293A cell line was used as a control. The studies described below used these two mammalian cell models of GLDC deficiency.

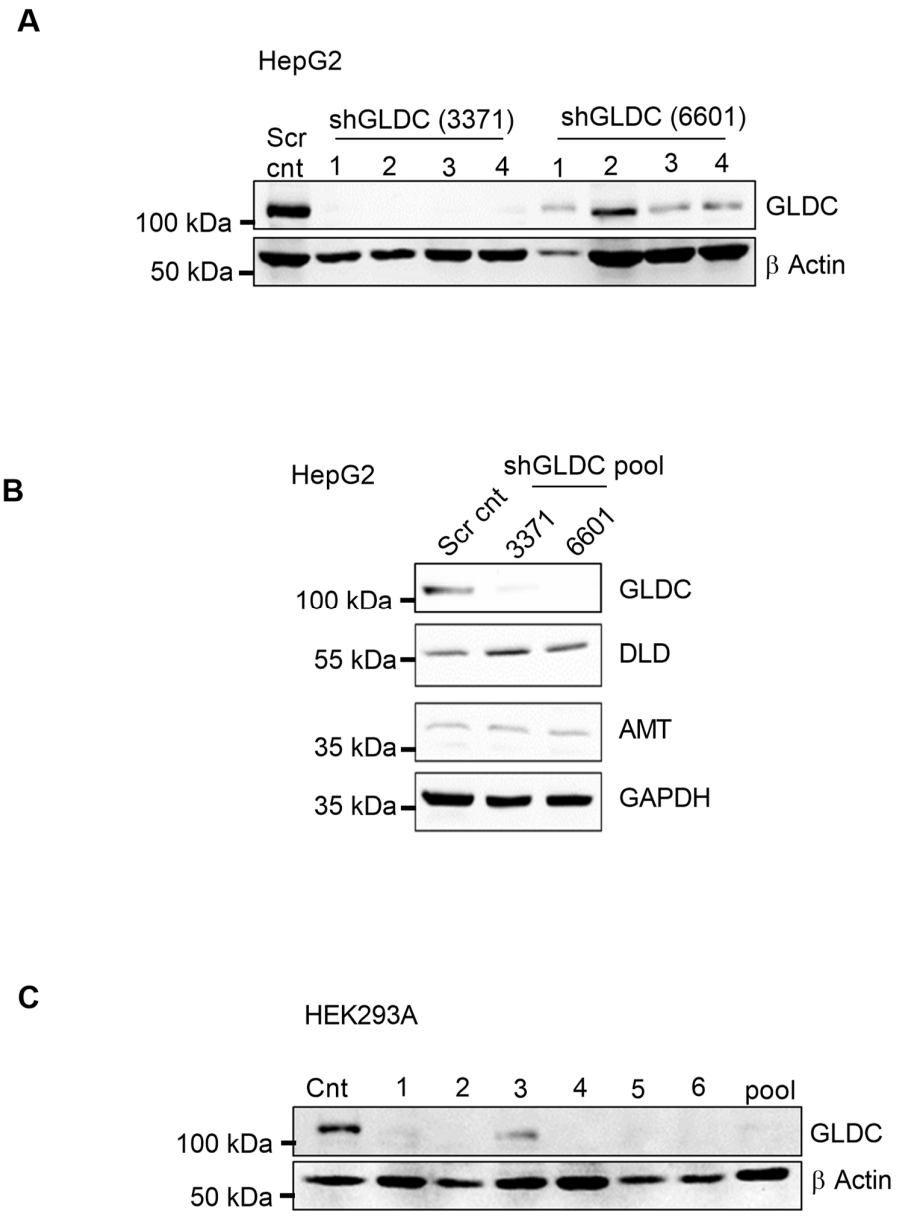

Figure 4.3: Establishment of stable cell lines with reduced GLDC expression
Western blot analysis to determine (A) GLDC protein expression following shRNA-mediated knockdown of GLDC in HepG2 cells. Two shRNA constructs (3371 and 6601) were used for targeting GLDC. Individual clones with stable GLDC knockdown from each construct selected on 2 µg/ml puromycin were analyzed. Actin was used as loading control. (B) GLDC, DLD and AMT protein expression following shRNA-mediated knockdown of GLDC in HepG2 cells. Heterogenous pool with stable GLDC knockdown from 3371 and 6601 shGLDC constructs selected on 2 µg/ml puromycin were analyzed. GAPDH was used as a loading control. (C) GLDC protein expression following Crispr/Cas9 genome editing. Independent cell clones selected on 1 µg/ml puromycin were examined. Actin was used as a loading control.

4.3.2 **GLDC is required for maintaining cellular redox balance.**

An important metabolic fate of glycine is as a component of the antioxidant glutathione. Glutathione is a tripeptide composed of cysteine, glutamate, and glycine, and the addition of glycine to the cysteine-glutamate di-peptide is the final step in the synthesis of glutathione. Given the key role of GLDC in regulating glycine availability, we initially hypothesized that elevated GLDC enzyme levels would lead to a reduction in glycine availability and consequent reduction in the rate of glutathione synthesis. With this same reasoning, reduced GLDC activity would increase glycine levels and potentially stimulate glutathione synthesis. To test this hypothesis, we measured total glutathione levels in cells with suppressed GLDC enzyme levels. Contrary to our expectations, knockdown of GLDC by shRNA in HepG2 cells (Figure 4.4 A), or by Crispr/Cas9 mutagenesis in HEK293A cells (Figure 4.4 B), actually resulted in a significant reduction in total glutathione production; 1.9-fold (P=0.004) and 1.8-fold, (P=0.025) respectively (Figure 4.4 C and D). These unexpected results suggest that in these experimental conditions cellular glycine levels are already in excess with regard to glutathione synthase activity. A potential explanation for these surprising findings is that product(s) of GLDC activity is required for glutathione synthesis. The most likely possibility is that the methylene tetrahydrofolate produced by GLDC action enters the folate-methionine-transsulfuration pathway, which is required for glutathione synthesis (Figure 4.2).

To determine if the observed reduction of glutathione synthesis affected cellular oxidative stress, we measured reactive oxygen species (ROS) levels in the HepG2 cells with shRNA knockdown of GLDC. Intracellular ROS levels were measured using the CM-H_2DCFDA dye which is a probe for general ROS levels. We also included a pro-oxidant (tert-butyl hydroperoxide) treatment to determine if the GLDC knockdown reduced the cell's ability to mount a protective glutathione-mediated response.

As predicted from the glutathione experiments described above, the endogenous levels of ROS were higher in the HepG2 GLDC knockdown clone, 1.76 fold (P=0.0003) (Figure 4.4 E, left-hand bars) as compared to the control cells.

The scrambled control HepG2 cells did not show an increase in ROS species when challenged with 150 μM of tert-butyl hydroperoxide, indicating that they could mount a compensatory antioxidant (protective) response (Figure 4.4 E). The GLDC knockdown cells showed an exacerbated response to the additional stress, with an increase in ROS of 79.4 % (P=0.0002) after tert-butyl hydroperoxide treatment. Similar results were obtained using the HEK293A GLDC knockout cell line. In these cells, the reduced glutathione levels induced by the GLDC knockout resulted in ROS elevation only when an external oxidative stress was applied (Figure 4.4 F). Together these results indicate that the GLDC-mediated changes in glutathione production resulted in increased ROS levels and susceptibility to oxidative stress.

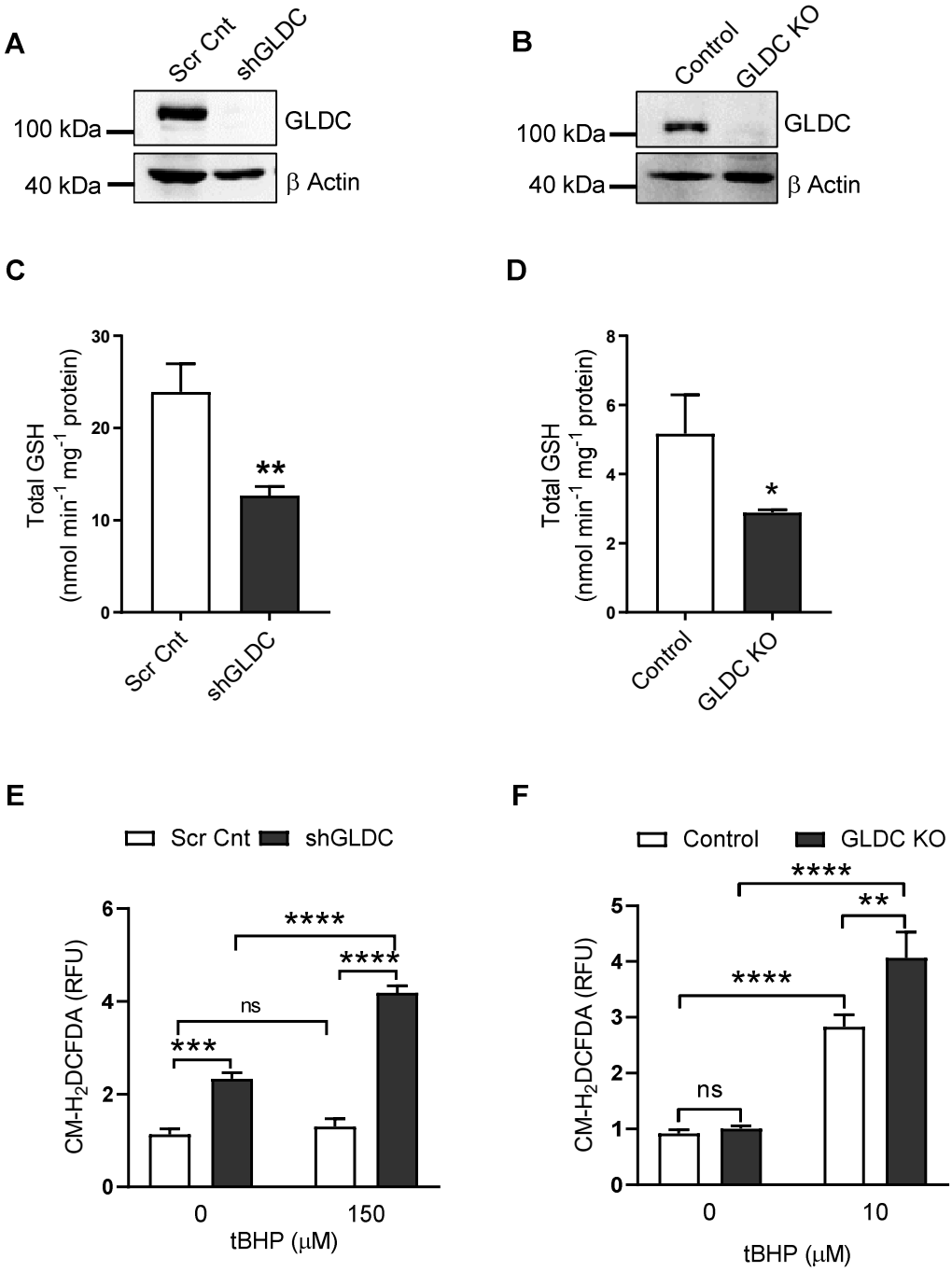

Figure 4.4: GLDC is required for maintaining cellular redox balance
(A) Western blot of HepG2 scrambled control (scr cnt) and GLDC stable knockdown (shGLDC) cell lines (B) HEK-293A control cell line and CRISPR/CAS9 mediated GLDC stable knockout (GLDC KO) cell line. In (A, B), β actin was used as a loading control. (C, D) Quantitative determination of total glutathione levels using an enzymatic recycling method in (C) HepG2 scrambled control and GLDC stable knockdown clone and (D) HEK293A control and GLDC stable knockout clone. Total glutathione levels were normalized to total protein. Error bars,

mean ± s.d.; *P<0.05, **P<0.01. The results were confirmed with 3 independent experiments. (E,F) Quantitative measurement of general intracellular oxidative stress in (E) HepG2 scrambled control and shGLDC stable cell line, and (F) HEK293A control and GLDC stable knockout cell line using CM-H_2DCFDA normalized to total protein using a fluorescence-measurement based plate reader. The cells were either treated with vehicle or tBHP at the indicated concentrations for 4h prior to dye addition. The results were confirmed with 2 independent experiments. Error bars, mean ± s.d.; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.3 **GLDC mediates cAMP-dependent stimulation of glutathione production.**

The experiments described above showing that GLDC knockdown reduces glutathione production, predict that elevated GLDC levels or activity would increase glutathione levels. We tested this possibility in primary rat hepatocytes in which GLDC levels were induced by treatment with db-cAMP. siRNA-mediated GLDC knockdown efficiency achieved in these cells was 68% (P=0.0164) (Figure 4.5 A). We verified GLDC knockdown as well as db-cAMP induction at the protein level (Figure 4.5 B). In these primary hepatocytes, db-cAMP treatment induced GLDC mRNA levels (4.44 fold, P=0.0005) (Figure 4.5 A) and resulted in a significant increase in total glutathione levels (Figure 4.5 C). This cAMP-mediated increase in glutathione production was dependent on GLDC since shRNA knockdown of GLDC (Figure 4.5 A and B) abolished the induction of glutathione production (Figure 4.5 C). These findings confirm that GLDC activity is linked to glutathione production.

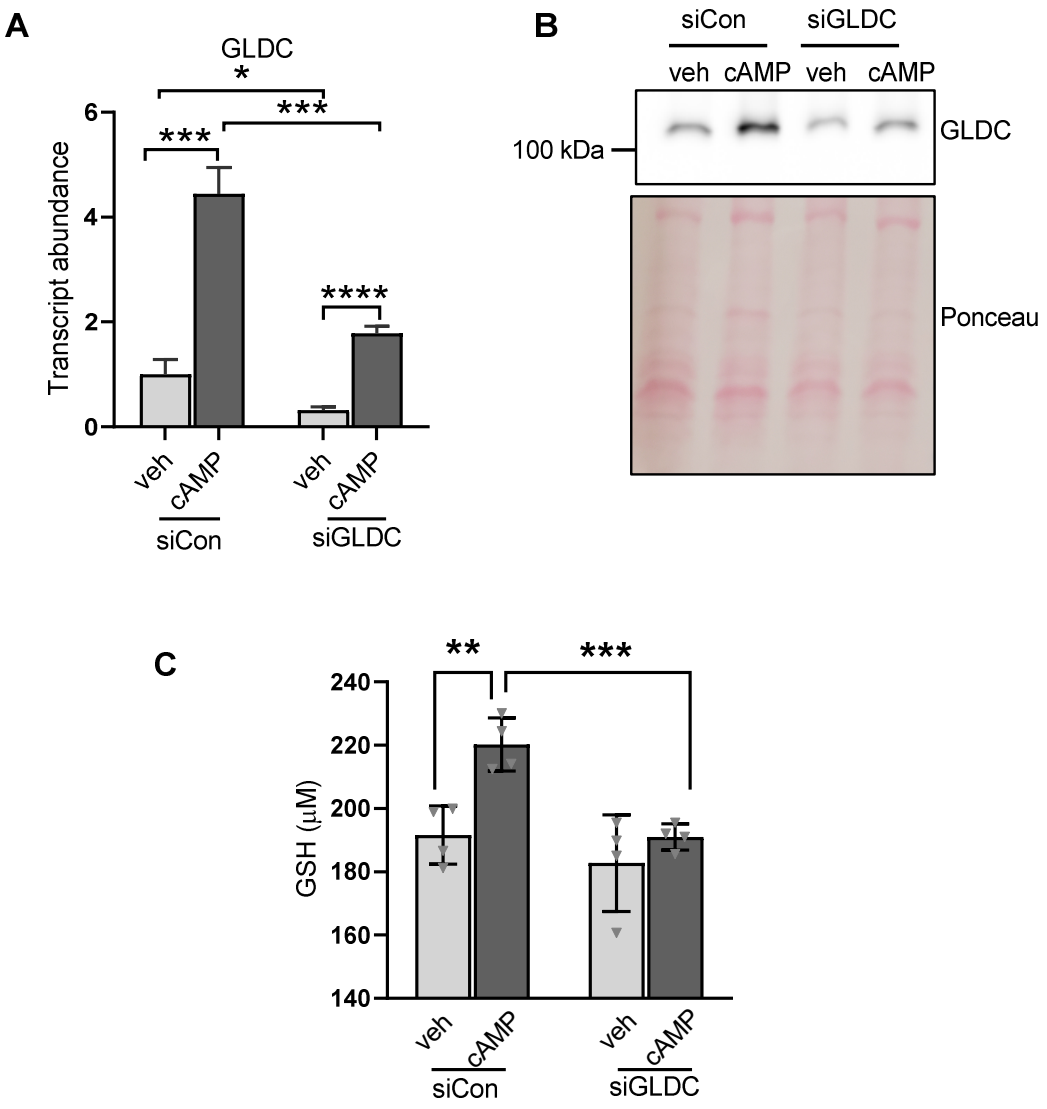

Figure 4.5: GLDC mediates cAMP-dependent stimulation of glutathione production
(A, B) Verification of knockdown efficiency of siControl and siGLDC in rat primary hepatocytes either treated with vehicle (veh) or cAMP by (A) RT-qPCR for transcript abundance and by (B) Western blotting for protein levels normalized to Ponceau staining of the membrane. (C) Rat primary hepatocytes transfected with either siControl or siGLDC and treated with cAMP for 18h and total glutathione production measured by luciferase-based GSH assay. Error bars, mean ± s.d.; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.4 **Additional effects of GLDC knockdown: cell proliferation and mitochondrial respiration.**

As mentioned above, GLDC activity could increase energy output in cells via production of NADH. Thus, reduced GLDC could potentially affect energy requiring cellular processes, such as proliferation. In fact, cells with loss of GLDC have been reported to display reduced cellular proliferation rates (86, 147, 152, 153). To study the regulatory effect of GLDC on HepG2 cell proliferation, 25000 cells from one clone each isolated from the two shGLDC vectors (3371 and 6601) were plated into 6-cm plates and grown in complete media. The number of cells present after 4 days was then counted using a hemocytometer. Consistent with reports in other cell lines, knockdown of GLDC in HepG2 cells reduced cellular proliferation rates (Figure 4.6 A).

Since NADH produced by GLDC can donate electrons to the mitochondrial electron transport chain and promote mitochondrial respiration, it is possible that knocking down GLDC would impair mitochondrial respiration. We therefore measured the basal oxygen consumption rate (OCR) of HepG2 scrambled control cells and GLDC 3371 targeting vector derived clones #1 and #3 HepG2 cells on a Seahorse analyzer. The relative OCR of GLDC knockdown clone #1 was decreased by 20% (P=0.0194) and in clone #3 decreased by 20% (P=0.0067) relative to scrambled control (Figure 4.6 B). These findings are consistent with the possibility GLDC activity is required for full mitochondrial respiratory function.

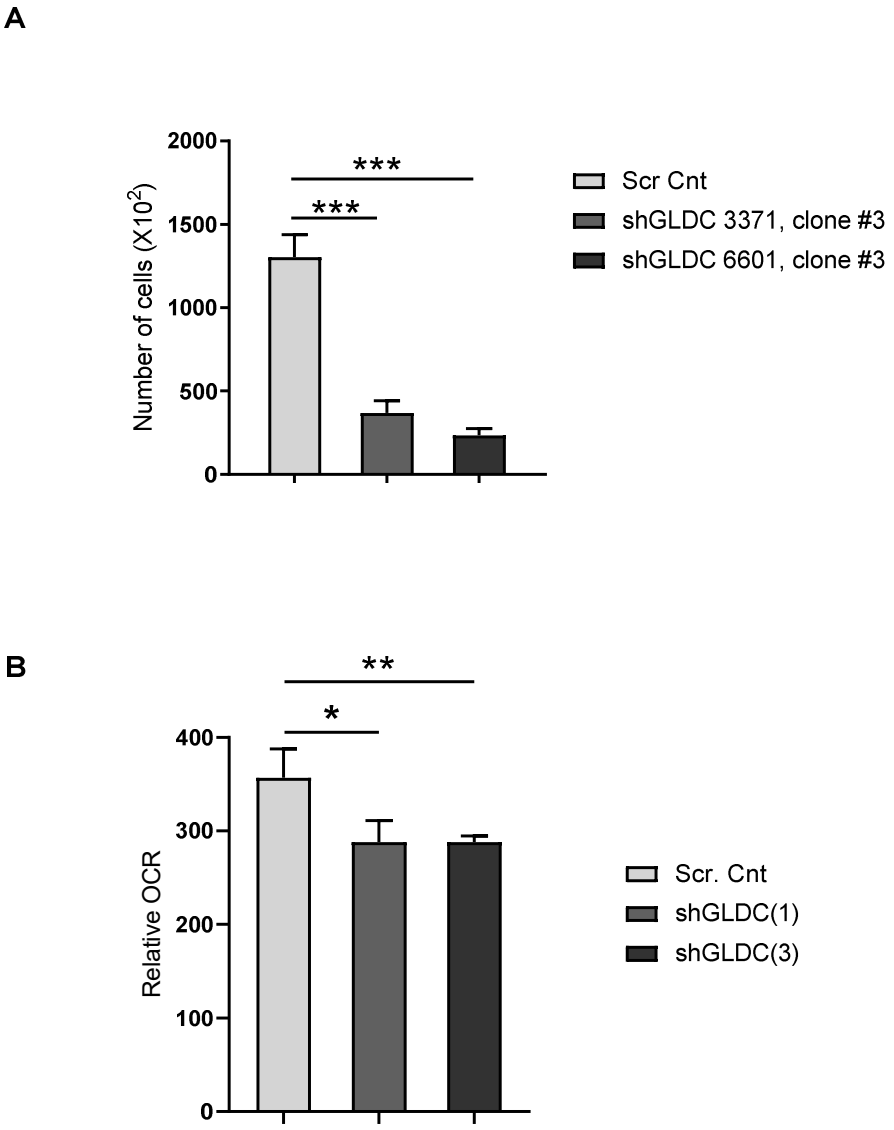

Figure 4.6: Loss of GLDC decreases cell proliferation and relative oxygen consumption rate in HepG2 cells.

(A) Cell proliferation rate of scrambled control and two individual clones isolated using shGLDC 3371 targeting construct. Cells were grown in DMEM/high glucose media. (B) Relative basal oxygen consumption rate of scrambled control and two individual clones isolated using shGLDC 3371 targeting construct. Cells were grown in DMEM/high glucose media. Data are presented mean $\pm$ s.d. *P<0.05, **P<0.01. (Student's t-test compared to scrambled control).

4.4 Discussion

In this chapter, we utilized cell models to study the physiological effects of altered GLDC expression. As presented in the introduction to this chapter (Section 4.1), a primary effect of reduced or increased GLDC expression *in vitro* and *in vivo* is an increase or decrease in glycine levels, respectively. The goal of the work described in this chapter was to determine if there were other physiological outcomes of altered GLDC expression in addition to changes in glycine levels. In particular, we proposed that given the requirement for glycine in glutathione biosynthesis, a stimulation of GLDC would reduce the available glycine pool and limit the ability of the cell to synthesize glutathione. Our finding that GLDC knockdown produced the opposite effect disproved this original mechanistic hypothesis and suggested that there was some other less well-understood link between GLDC and glutathione production.

Using established stable cell lines with reduced GLDC expression we were surprised to observe that reduced GLDC expression caused a suppression of total glutathione levels and an increase in ROS levels. One of the reasons our initial hypothesis was incorrect might be related to the *in vitro* cell culture circumstances. These *in vitro* experiments were performed in growth media containing 300 μM glycine, well in excess with regard to the enzyme glutathione synthase, which adds glycine to the cysteine-glutamate dipeptide to form glutathione. It would be interesting to perform total glutathione measurements with varying glycine concentrations in the growth media.

Our findings led us to propose that the product of glycine cleavage by GLDC, 5,10-meTHF, is an input into the one-carbon metabolism cycle. One of the outputs of this system is glutathione production through the transsulfuration pathway (based on figure 1.3 and 1.4), which could be the mechanistic link between GLDC and glutathione production.

These results raise the question- ***Why does a reduction in GLDC expression lead to reduced glutathione production?*** The glycine cleavage system is linked with the one-carbon metabolism which is comprised of the folate cycle (mitochondrial), methionine cycle (cytosolic), and the transsulfuration pathway (cytosolic). One carbon metabolism directly controls serine, glycine, and methionine levels and indirectly controls cysteine levels. Homocysteine transfers sulfur to serine to make cysteine via the transsulfuration pathway. The notable aspect of our findings is that reducing the levels of a mitochondrial enzyme (GLDC), caused a significant reduction in a product being generated in the cytosol (glutathione), even in the presence of excess glycine. Amplification of GLDC expression in certain cancers suggest that the mitochondrial glycine cleavage system is important as a source of one carbon units. Loss of function mutations in glycine cleavage system components cause an elevation in embryonic tissue glycine levels (77). We already know *in vitro* GLDC knockdown leads to an increase in glycine. This implies that the decline in glutathione levels observed upon GLDC knockdown are not due to a deficiency of glycine for the last step of glutathione biosynthesis. Labuschagne et al. (2015) showed glutathione levels were relatively unaffected across varying concentrations of serine and glycine in DMEM media containing 17 mM glucose. These cells had functional glycine cleavage system as well as other serine/glycine biosynthetic enzymes which implies the cells can generate serine and glycine from glucose in the media, if needed (154). These results were also supported by Ducker et al. (2016) who showed in HEK293T cells with functional serine/glycine pathway enzymes, glutathione levels are unaffected in glycine depleted media. When they disrupted the flow of mitochondrial one-carbon groups by knocking out either SHMT2 or MTHFD2, there was a decline in glutathione levels upon glycine depletion but not in glycine replete conditions (155). Leung et al. (2017) showed a decline in the relative abundance of me-THF in embryos carrying GLDC and MTHFR loss-of-function alleles (156). Although there is no direct evidence that this occurs, but with these

supporting evidences, we can predict that the decline in glutathione levels after GLDC knockdown is due to a reduction in the one-carbon groups generated by glycine degradation. The one carbon groups link to the transsulfuration pathway via cysteine biosynthesis and therefore contribute to glutathione levels. Formate, the terminal product of the mitochondrial folate metabolism, is shuttled to the cytosol to meet the one-carbon demands of the cytosolic folate and methionine pathways (83). It would be interesting to see if formate is able to rescue some of the effects of GLDC knockdown on total cellular glutathione levels.

Our results in rat primary hepatocytes showing that a cAMP-mediated increase in GLDC was associated with increased glutathione production is in contrast to a previous study by Lu et al., (1991), which showed a decline in cellular GSH levels with db-cAMP treatment in rat hepatocytes (157). The differences in growth medium composition and GSH measurement method between their study and ours might account for this discrepancy. Our findings linking GLDC to glutathione production were consistent across multiple cell lines and knockdown methods. Also in contrast to our findings, a previous study has shown that knocking down GLDC in Huh7 cells resulted in an increase in total glutathione levels and a decreased GSH/GSSG ratio in their experimental system (158), essentially the opposite of our results. We saw a decrease in total glutathione levels after knocking down GLDC in HepG2 and HEK293A cells but were unable to effectively measure GSH/GSSG ratio. However, similar to Zhuang, Li et al. (2018), we saw an increase in reactive oxygen species. It may be worth noting that the effect of GLDC knockdown on cell migration, invasiveness, and metastasis by (158) was in contrast to most reported effects of GLDC on cancer progression (159).

In summary, the work presented in this chapter identified a previously unidentified link between a mitochondrial enzyme, GLDC, and the antioxidant glutathione possibly connected via mitochondrial folate-mediated one-carbon units from glycine cleavage supporting cytosolic glutathione production and thereby redox balance.

CHAPTER 5 - FINAL CONCLUSIONS AND FUTURE DIRECTIONS

The overall goals of my thesis work were to provide a mechanistic explanation for the longstanding observation that glycine levels are suppressed in obesity, diabetes, and related metabolic diseases, and to explore the possibility that altered glycine metabolism played a role in the development of these syndromes. The work was organized around the hypothesis that GLDC, the rate-limiting enzyme in glycine degradation, is a central regulator of glycine metabolism responsible for determining glycine levels and linking glycine metabolism to physiological pathways related to metabolic disease. For this reason, we (1) examined the expression of GLDC under metabolic and nutritional stress conditions, (2) identified the hormonal regulation and signaling pathways involved in the transcriptional regulation of GLDC, (3) determined the impact of altering GLDC expression on cellular physiology.

Our data demonstrated that GLDC expression is altered in mouse models of type 2 diabetes and by fasting and that hormones associated with metabolic and nutritional regulation (glucagon and insulin) are key regulators of GLDC gene expression. We speculated that there exists a link between GLDC and gluconeogenesis via one of the products of glycine cleavage-NADH (See Chapter 3 'discussion'). To confirm this idea, future work should include measurement of ATP levels, mitochondrial membrane potential, and NAD^+/NADH ratio in cells with altered GLDC levels after db-cAMP treatment. It will be interesting to measure glucose production in cells with GLDC knockdown after db-cAMP treatment. Cells can be provided with either lactate or pyruvate as substrates the night before and then treated with db-cAMP for 8h to stimulate gluconeogenesis. We expect a decline in glucose production in cells with reduced GLDC levels.

Finally, we demonstrated that GLDC activity is linked to glutathione production. We think that altering GLDC causes metabolic rearrangements and an alteration in the one-carbon flux through the mitochondrial folate pathway (see figure 1.4), the interlinked cytosolic

methionine cycle, and consequently in the activity of the cytosolic transsulfuration pathway which generates the cysteine required for glutathione biosynthesis (see figure 1.3). To confirm this idea, future work using metabolic flux analysis needs to be conducted by replacing glycine in growth media by labeled glycine (U-^{13}C glycine) and then monitoring the kinetics of labeled glycine incorporation into cells and the downstream metabolic product, glutathione. It will be interesting to measure if formate is able to rescue the effects of GLDC suppression *in vitro* by providing one-carbon units that can be freely exchanged between the mitochondrial and cytosolic folate pathway. Given the crucial role of glutathione in normal physiology and in metabolic pathologies, this functional link between GLDC and glutathione that we identified suggests a pathway by which alterations in glycine catabolism could play a functional role in the development of diabetes and related metabolic diseases. Our data also demonstrated that knocking down GLDC influenced various cellular physiology parameters. Knocking down GLDC led to a decrease in cell proliferation and mitochondrial oxygen consumption rate. In cells expressing stable reduction in GLDC expression, total glutathione levels were significantly reduced with an expected concomitant increase in cellular ROS levels.

Going forward, a careful assessment of the altered GLDC expression *in vivo* will be required to understand if there exists a causal link between GLDC expression, glycine levels, redox balance, and susceptibility for metabolic disease development and/or progression. Briefly, for this assessment, two models will be used: (1) WT-GLDC overexpression, and (2) shRNA-mediated GLDC knockdown. Recombinant adeno-associated viruses with liver tropism will be used for hepatic GLDC overexpression or knockdown. 4 weeks after viral injection, the mice will be placed on either a diabetogenic high-fat diet (65% calories from fat) or a low-fat diet (10% calories from fat) for 16 weeks. Body composition (Echo-MRI), glucose tolerance, insulin tolerance, and whole-body energetics (metabolic cages) will be measured at regular intervals. Glycine levels will be measured by mass spectrometry. Total glutathione and

GSH/GSSG ratio will be measured both in blood and tissue using commercial kits. We expect to observe a reduction in glycine, an increase in total glutathione with GLDC overexpression and vice versa. It would be interesting to see if and how altering hepatic GLDC and then inducing diabetes would affect the progression of the metabolic disease in a whole-animal physiology.

Our results add to our understanding of how glycine metabolism is integrated into the complex metabolic networks responsible for general physiological homeostasis and lay the groundwork for future studies exploring the mechanistic detail of the metabolic relationships we have identified. The long-term hope is that the information generated here and in follow-up studies will suggest pathways for the development of novel therapeutics to treat or prevent type 2 diabetes and related diseases.

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ABSTRACT

HORMONAL REGULATION OF GLYCINE DECARBOXYLASE AND ITS METABOLIC OUTCOMES

by

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Advisor: Dr. Todd Leff and Dr. Jian Wang

Major: Pathology

Degree: Doctor of Philosophy

The amino acid glycine is involved in generation of multiple critical metabolites including glutathione, heme, and creatinine. Interestingly, in both humans and rodents, circulating glycine levels are significantly reduced in obesity, glucose intolerance, type II diabetes and non-alcoholic fatty liver disease. The glycine cleavage system is the predominant glycine degradation pathway in humans. The rate-limiting enzyme of glycine cleavage system is glycine decarboxylase (GLDC), and loss-of-function mutations of GLDC cause hyperglycinemia. Here, we show that GLDC gene expression is upregulated in livers of mouse models of diabetes and diet-induced obesity as well as in the fasted state in normal animals. In exploring the hormonal signals that mediate these regulatory events we found that both glucagon and insulin stimulated GLDC gene expression. In primary rat hepatocytes, GLDC expression was strongly stimulated by glucagon and cAMP, and mildly with insulin while in a rat hepatoma cell line, insulin strongly stimulated GLDC expression as compared to cAMP. We identified both cAMP-response element binding protein 1 (CREB1) and activating transcription factor 1 (ATF1) as mediators of the glucagon regulation, while insulin responsive transcription factor sterol regulatory element binding protein 1c (SREBP1c) mediated the insulin stimulatory effect on GLDC transcription. We also observed that altering GLDC

expression levels strongly affected intracellular glutathione levels and levels of reactive oxygen species (ROS). Our findings suggest that the hormonal regulation of GLDC may contribute to a compensatory increase in glutathione production as a defense against metabolic disease-associated oxidative stress.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

Ph.D., Molecular Pathology of Human Disease, Wayne State University School of Medicine, Detroit, MI, USA	2013-present
M.Sc. (5-year Integrated), Biotechnology, Institute of Bioinformatics and Biotechnology, Pune, Maharashtra, India	2007-2012

FELLOWSHIPS

1. ASBMB Graduate student and postdoctoral travel award for Experimental Biology, San Diego, CA 2020
2. Werner U. Spitz, M.D Endowed Scholarship in Pathology, Dean's Scholarship Committee, Wayne State University School of Medicine 2018
3. Summer Dissertation award, The Graduate school, Wayne State University 2018
4. National Institute of Health's (NIH) Broadening Experiences in Scientific Training (BEST) Phase III Career Exploration Opportunity Recipient, Technology commercialization office, Wayne State University 2017

AWARDS

1. **Third** place, Graduate Student and Postdoctoral research symposium 2020
2. **First** place, Chuan-Pu Lee, Ph.D. Endowed Graduate Student Research Presentation Day, Detroit, MI, US. 2018
3. **Second** place, Michigan Regional Chapter of the Society of Toxicology Fall Metabolic Health Symposium, Detroit, MI, US. 2017
4. **Second** place, Graduate student and postdoctoral research symposium, Detroit, MI, US. 2017
5. **Honorary mention** category, Graduate Student Research Day, Detroit, MI, US. 2017

PUBLICATIONS

1. Leff T, Stemmer P, Tyrrell J, **Jog R**. Diabetes and Exposure to Environmental Lead (Pb). Toxics. 2018; 6(3):54.
2. Matkar, P. N., Cao, W. J., Chen, H. H., Civitarese, R., **Jog, R.,** and Bugyei-Twum, A. Rac1: an emerging player in stretch-stimulated glucose transport. Journal of Physiology 2015, 593(8): 1771-1772.
3. **Jog, R.**, Chen, G., Leff, T., and Wang, J. Threonine catabolism and epigenetic control of mouse embryonic stem cells. Handbook of Nutrition, Diet and Epigenetics (2017).
4. **Jog, R.,** Chen, G., Wang, J., and Leff, T. (2019) Hormonal regulation of glycine decarboxylase and its impact on cellular physiology. (under preparation).
5. Tyrrell, J., Hafida, S., Stemmer, P., Chagas, C., **Jog, R.** & Leff, T. (2019) Lead (Pb) Exposure Exacerbates Metabolic Abnormalities in Mouse Models of Diabetes. (under preparation).