Control of glucose homeostasis through
ubiquitin-specific protease 2

by

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A dissertation submitted in partial fulfillment
Of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
2011

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Professor Ormond A. MacDougald
Professor Alan R. Saltiel
Professor Jessica Schwartz
To my Mom,

for always believing in me.
Acknowledgements

I am forever indebted to my mentor, Dr. Jiandie Lin, for allowing me the opportunity to work in his lab. It might not have always been easy but I truly appreciated his patience and guidance. I would also like to thank Dr. Siming Li for her help with experiments and her willingness to listen. I truly believe she is the heart and soul of the lab, and without her the lab would not operate as smoothly as it does. This work never would have been completed without the help and support of my colleagues in the Lin lab, including Zhouxian Meng, Fang Fang, Guoxiao (Grace) Wang, Di Ma, research technician Lei (Layla) Yu, as well as past lab members Chang Liu and Carlos Hernandez.

I would also like to thank my friends; Dave, Mike, Dan, Ryan, Superfly, Ferris, Bev, Chou, Vanilla, Justin, Kelly, Jen, Ashley, Dehudy and Taylor for making life up here in Michigan fun and entertaining. I will always remember the football tailgates as well as the numerous rafting trips we took down the Huron. None of this would have been possible without the support of my friends back home. To Jake, CB, Timmy, Stew, Zenny, and Rutt thanks for making me laugh to the point where it hurts. River Crew, assemble!
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<td>Acetyl-CoA carboxylase 2</td>
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<td>Brain and muscle ARNT-like 1</td>
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<td>CK1(\delta/\epsilon)</td>
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<td>Deubiquitinating enzymes</td>
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<td>HPA</td>
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<td>HSD1β11 or HSD1</td>
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<td>LKO</td>
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<td>MDM2</td>
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<td>Metabolic syndrome</td>
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<td>mRNA</td>
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<td>PEPCK</td>
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<td>s.e.m</td>
<td>Standard error mean</td>
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<td>TSH</td>
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<td>Ub</td>
<td>Ubiquitin</td>
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<td>USP</td>
<td>Ubiquitin-specific protease</td>
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<tr>
<td>VLCFAs</td>
<td>Very-long chain fatty acids</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber time</td>
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Abstract

Hepatic gluconeogenesis is important for maintaining steady blood glucose levels during starvation and through light/dark cycles. The regulatory network that transduces hormonal and circadian signals serves to integrate these physiological cues and to adjust glucose synthesis and secretion by the liver. However, questions remain as to what factors are involved in coordinating circadian signalling and metabolic regulation. We identified ubiquitin-specific protease 2 (USP2) as an inducible regulator of hepatic gluconeogenesis that responds to nutritional status and clock signalling. Adenoviral-mediated overexpression of USP2 in the liver promotes hepatic glucose production, whereas RNAi knockdown of this factor results in hypoglycemia due to impaired hepatic gluconeogenesis. USP2 is required for maintaining diurnal glucose homeostasis during restricted feeding. Elevated hepatic gluconeogenesis exacerbates the development of hyperglycemia in diabetes. In vivo gain- and loss-of-function studies demonstrated that USP2 regulates systemic glucose metabolism in insulin resistant states through modulation of hepatic glucocorticoid signalling and the gluconeogenic program. Interestingly, we found that USP2-45 is localized to the peroxisome in culture primary hepatocytes; however, what role this plays in USP2 function remains unclear.
USP2 mRNA levels are regulated by both fasting and circadian signals. To identify key factors involved in USP2’s regulation I employed a gene reporter assay. From these experiments I identified the PPAR gamma co-activator 1 (PGC-1) family of transcription factors as positive regulators of USP2 expression. This activation was mediated in part by circadian regulated transcription factors: estrogen-related receptor gamma (ERRγ) and hepatic nuclear factor 4-alpha (HNF-4α) and antagonized by E4 binding protein 4 (E4BP4, Nfil3). Together, my work delineates a novel pathway that links hormonal and clock signalling to hepatic gluconeogenesis and glucose homeostasis.
Chapter 1

Introduction

1.1. Emerging Health Risks Associated with Increases in Obesity

An increased sedentary life-style as well as excessive consumption of energy-rich foods has led to the increase in the prevalence of obesity worldwide. Obesity is a growing epidemic in the industrialized world as well as developing countries. It is estimated that 1.1 billion people are diagnosed as over-weight with a body-mass index (BMI) over 25kg/m$^2$, of which 300 million are considered obese (BMI = 30 kg/m$^2$ or greater). Additionally, it is estimated that 155 million children are over-weight or obese worldwide [1, 2]. Also increased BMI is associated with decreased life expectancy. Studies done in the UK estimate that a BMI of 25 kg/m$^2$ decreases the life expectancy of adult men by two years and projects up to a five year decrease in life expectancy by 2050 [1, 3]. In the United States approximately 280,000 deaths annually are
attributed to obesity and the direct and indirect costs of obesity were estimated at $123 billion for 2001 [2, 4]. Thus, obesity is not only a burden to the individual but it is also damaging to society as a whole. It is therefore necessary to elucidate not only the epidemiological factors involved in obesity but also the molecular factors that underlie the physiological state. Herein we will look at the morbidities of obesity specifically focusing on insulin resistance and regulation of glucose homeostasis.

1.1.1. Metabolic Syndrome and Type 2 Diabetes (T2DM)

Obesity, in this case centralized obesity, is just one aspect of a cohort of conditions termed ‘metabolic syndrome’ (MetS) which includes: excessive adiposity, insulin resistance, dyslipidemia and increased blood pressure [5]. Furthermore, this cluster of conditions leads to an increased risk of cardiovascular disease (CVD) as well as a 5-fold increase in developing type 2 diabetes mellitus (T2DM) [6].

In humans, T2DM is a polygenic, heterogeneous disease that results in the loss of insulin sensitivity (insulin resistance) in peripheral tissue (liver, muscle, and adipose tissue), characterized by hyperglycemia, hyperinsulinemia and hypertriglyceridemia. Insulin resistance in T2DM occurs post-receptor, referring to a dysfunction in down-stream insulin signaling rather than dysfunction in release of the hormone. While the exact diagnostic parameters vary amongst different organizations, a core set of criteria are needed for diagnosis of T2DM.
During post-prandial food absorption insulin stimulates glucose uptake in muscle and fat tissue via the glucose transporter, GLUT4. Insulin also suppresses glucose production in the liver (termed hepatic glucose production or HGP) and increases glucose storage in the form of glycogen. In the initial stages of T2DM, decreased insulin sensitivity in peripheral tissues, primarily in muscle and fat, results in excessive amounts of circulating glucose. To compensate the β-cells of the pancreas increase production and release of insulin to aid in disposal of the excess glucose. As the disease progresses insulin is less able to repress hepatic glucose production resulting in further increases in circulating glucose. Finally, β-cells start to fail, resulting in diminished insulin secretion and a breakdown in glucose homeostasis [7, 8].

1.1.2. Role of Liver in maintaining Glucose Homeostasis

As stated above, one aspect of hyperglycemia in T2DM is an increase in glucose production from the liver. Glucose is an important energy source for many cells throughout that body and therefore must be maintained within a critical range. Several pathways control the flux of glucose including the rate of consumption and absorption of carbohydrates, as well as the disposal and utilization of glucose in peripheral tissues (ie muscle and fat). Glucose homeostasis is also highly regulated by the liver. The liver in general has a tremendous affect on glucose homeostasis involving balances between glucose storage (glycogen) and breakdown (glycogenolysis & glycolysis) as well as de
*novo* glucose production termed gluconeogenesis. In must be pointed out that gluconeogenesis also occurs in kidney tissue. However, it is still being debated as to how much this renal glucose output contributes to overall glucose production - in humans the estimate ranges from 5-23%. During times of starvation, glycogen breakdown (glycogenolysis) in the liver aids initially in maintaining blood glucose levels, which are regulated in part by increased circulation of the hormone glucagon – released from the α-cells of the pancreas. However, as glycogen stores become depleted, the liver increases the rate of gluconeogenesis using lactate, glycerol and gluconeogenic amino acids as precursor substrates.

Hepatic glucose production is a tightly regulated process including hormonal input (insulin and glucagon as well as others) and the transcriptional and non-transcriptional regulation of several rate-limiting enzymes including Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose-6-phosphatase (G6Pase). PEPCK is the first rate-limiting step in gluconeogenesis and catalyzes oxaloacetate to phosphoenolpyruvate (PEP). While G6Pase catalyzes the final step producing free glucose from glucose-6-phosphate (G6P) [9]. Since glucose is passively transported into the bloodstream via GLUT2, G6pase can be thought of as the gatekeeper of glucose production in the liver. Disruption of PEPCK or G6Pase has a dramatic affect on glucose homeostasis. Mutations in the G6Pase gene lead to glycogen storage disorder 1a (GSD1a) characterized by hypoglycemia and increase liver glycogen content [10]. G6pase null mice also display characteristics of GSD1a including hypoglycemia [11]. Additionally, liver
specific knock-out of PEPCK leads to marked impairment of hepatic gluconeogenesis while total body knock-out causes severe hypoglycemia and resulting death [12, 13]. While there is some evidence that PEPCK might be allosterically regulated, it is generally accepted that alterations in PEPCK and G6Pase mRNA levels control the overall activity of the enzymes and therefore gluconeogenesis. This tight transcriptional regulation can be observed best by the multitude of transcription factors found to bind to proximal promoters of both genes [14, 15].

1.1.3. Glucocorticoid Signaling In Liver Tissue

Glucocorticoids (GCs) are a class of steroid hormones that regulate numerous physiological pathways including: glucose homeostasis, protein and lipid metabolism as well as a therapeutic role in anti-inflammation. Here we will focus primarily on GCs role in liver physiology. Indeed GCs have an important role in regulating gluconeogenic genes, PEPCK and G6Pase (Section 1.1.2), and therefore have been shown to oppose the action of insulin and decrease glucose utilization. To this end GCs have been categorized as catabolic hormones. The synthesis of glucocorticoids originates in the cortex of the adrenal gland, which is under the control of the hypothalamus-pituitary-adrenal (HPA) axis. In humans, glucocorticoids circulate as a protein-bound inactive precursor (cortisone) that can be converted into active hormone (cortisol) in tissues by 11β-hydroxysteroid dehydrogenase 1 (HSD11β1 or HSD1), an endoplasmic reticulum (ER)
membrane protein [16, 17]. Indeed the term glucocorticoid derives from the words; glucose, cortex and steroid [18].

The principle action of GCs is through its activation of the glucocorticoid receptor alpha (GRα, henceforth referred to as GR), a nuclear receptor that resides in the cytoplasm (or resides predominantly in the cytoplasm [19]) when unbound. Like many others in the nuclear receptor class, GR contains a co-activation domain in the N-terminal (NTD) which is required for interactions with other co-factors, a DNA binding domain (DBD) responsible for binding to its response element and a ligand binding domain (LBD) which is important for ligand activation as well as interactions with various co-factors. In the unbound state, GR is complexed with heat-shock proteins: hsp40, hsp70, and hsp90, as well as p23 and p60 [20]. Upon ligand binding GR dissociates from the inactive complex and translocates to the nucleus whereupon it binds, as a dimer, to its target response element, GRE (glucocorticoid response element; GGTACAnnnTGTTCT). Once bound to its response element GR recruits and interacts with co-activators such as CBP, SRC-1, p300/CBP and GRIP-1 increasing histone acetylation, opening up the chromatin and allowing for the recruitment of the RNA polymerase II, complex increasing gene transcription [21].

While glucocorticoid action can be thought of as a primary response on gene transcription, there can also be secondary responses to GC signalling via up-regulation of certain transcription factors or co-activators that propagate the GC signal. Furthermore, many genes not only require GR and a GRE for activation, but also rely on other transcription factors and binding sites for proper regulation.
This clustering of binding sites is referred to as a glucocorticoid responsive unit (GRU). This has been shown to be true for genes involved in hepatic gluconeogenesis, like PEPCK, which contains not only GR binding sites but also binding sites for liver enriched transcription factors HNF-4α, FoxA2, and C/EBPα [21].

Excess activity of glucocorticoid signalling has been implicated in the development of glucose intolerance in patients with Cushing’s Syndrome and also contributes to the pathogenesis of metabolic syndrome [22-25]. Tissue-specific activation of glucocorticoid signalling by transgenic expression of HSD1 leads to the development of key features of metabolic syndrome, including central obesity, glucose intolerance, and hypertension [26, 27]. On the contrary, HSD1 inhibitors, such as carbenoxolone (CBX), improve glycemic control in rodents as well as in humans, in part through attenuation of hepatic gluconeogenesis and glucose output [24, 28-31]. In fact, identifying new inhibitors for HSD1 is a hotbed of research in the type 2 diabetes field.

1.2. Circadian Rhythm

Circadian rhythms (Latin: Circa-, ‘approximately’ and -diem, ‘day’) are recurring biological processes such as: eating, sleeping, loco-motor activity and wakefulness that run roughly on a 24hr cycle. These physiological behaviors are coordinated by an intricate set of internal, endogenous, molecular “clocks” that maintain synchrony with environmental cues (e.g. light-dark cycles). Internal
molecular clocks (also known as circadian clocks) have been described in almost all forms of life on earth from prokaryotes to higher order eukaryotes such as mammals [32, 33]. This phenomenon has also been observed in primary cultures and immortalized cell lines like NIH3T3 cells [34-37]. Two important characteristics of these circadian clocks are 1.) the free-running period of 24hrs is temperature compensated, which is to say the clock does not run faster or slower with respect to the ambient temperature and 2.) biological clocks can be entrained (i.e. synchronized) to environmental stimuli such as light, feeding/fasting and temperature such that internal, or endogenous, time becomes predictive of external time [38, 39]. It is thought that these circadian rhythms evolved as a selective advantage for organisms to coordinate cellular energy metabolism with the environment, and temporally segregate mutually antagonistic systems (e.g. glycolysis/ gluconeogenesis) [38, 40-42]. Furthermore, mounting evidence suggests not only does circadian clock directly regulate metabolic processes, but also that metabolic parameters affect the actions and functions of circadian clocks [41, 43-47]. Because of this, circadian clocks and metabolic processes are thought to be intricately connected and not as simple as a linear relationship [42, 48].

1.2.1. Circadian Rhythm in Mammalian Systems

In mammals there is a hierarchical organization to circadian rhythm and coordination of physiological behaviors. There is a core molecular clock, or
master pace-maker, that is located in the superchiasmatic nucleus (SCN) of the hypothalamus [33]. This patch of roughly 10,000 cells is located above the optic nerve crossing that can be entrained by light cues relayed from melanopsin-containing retinal ganglion cells (retino-hypothalamic tract) [42, 49]. Thus this light entrain-able central clock is able to disseminate information to peripheral tissues to coordinate daily activities such as feeding (although there is evidence that a food entrain-able oscillator resides in the dorsomedial hypothalamic nucleus, DMH [50, 51]), wakefulness and loco-motor activity (Figure 1-1). Experiments in rodents, either through ablation or mutations studies, show the SCN to be the master pacemaker coordinating daily activities [33, 52-54]. It is also important to note that while ablation of this cluster of cells resulted in a loss of behavioral rhythms, it also resulted in loss of corticosterone (cortisone in humans) secretion from the adrenal gland. This suggests that not only does the SCN control locomotor activity but it also could play a role in proper maintenance of hormonal signaling [51].

Through molecular analysis it has been revealed that circadian clocks exist in most tissues and cell types within vertebrates – these are termed peripheral clocks. These peripheral clocks are not only entrained by cues from the central clock but are also highly entrain-able by nutrients [39, 55-57]. It is estimated that roughly 5-20% of genes in peripheral tissues display diurnal regulation at the transcriptional level and most of these genes are tissue specific [43, 46, 48, 58, 59]. Tissues such as the liver have inputs from the central clock regulating glucose homeostasis, but the liver has also been shown to contain a
robust molecular clock of its own. Many of the genes that oscillate in the liver involve metabolic pathways such as: glycolysis and gluconeogenesis, cholesterol and lipid homeostasis as well as xenobiotic metabolism. [41, 43, 45, 60].

1.2.2. Molecular Machinery Controlling Circadian Rhythm

The molecular underpinnings of the intracellular circadian clock reside in a series of interlocking transcription/translation feedback loops that results in a gene expression profile with a roughly 24h periodicity. It should be noted however, that it has recently been described that in some cell types a nucleus is not required for a properly functioning clock. The activating arm of this feedback loop is composed of a heterodimeric transcription factor complex, containing Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle Arnt-Like protein 1 (BMAL1), which are members of the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) protein family. This heterodimeric complex regulates Period (Per) and Cryptochrome (Cry) genes - via an E-box (5’ -CACGTG- 3’) element in the promoters of both genes. The products of these genes form dimers and translocate back to the nucleus to inhibit BMAL1:CLOCK transcriptional activity. Accumulation of the PER:CRY complex causes ubiquitin mediated proteasomal degradation of the PER:CRY complex, resulting in de-repression resetting the feedback loop allowing it to start again (Figure 1-2). The timing of the proteasomal degradation of PER and CRY is in part carried out by the casein family of kinases CK1δ and CK1ε. The layered nature of this
Figure 1-1. Integration of the central pacemaker of the SCN and peripheral clocks involved in metabolic regulation. Light signals derived from the retina are relayed via the RHT to the SCN. The SCN is then able to synchronize and entrain peripheral clocks by either modulating circulating hormones or by autonomic innervation. Also, feeding cycles are able to entrain the SCN but also plays a direct role in synchronizing peripheral clocks like that of adipose, liver and muscle tissue.
feedback loop allows for multiple points of input to fine-tune the running of the core clock. A second, well characterized feedback loop involves the binding competition of Retinoic acid receptor-related Orphan Receptor alpha (RORα), and REVerse ERythroBlastosis virus alpha (REV-ERBα) for the RORE in the Bmal1 promoter. RORα through interactions with Peroxisome Proliferator-Activated Receptor (gamma) Coactivator 1-alpha (PGC-1α), activate transcription of BMAL1 where as REV-ERBα represses transcription [61]. This alternating occupancy of the RORE is made possible by the rhythmic expression of REV-ERBα – driven by the BMAL1:CLOCK complex [40, 42, 51, 62]

1.2.3. Linking Circadian Rhythm and Metabolic Disorders

Mounting evidence suggests that perturbations in feeding habits cause a dramatic effect on clock signaling and clock-controlled output behaviors. Recent studies show that diets rich in fat have a profound effect on rhythmic behaviors such as locomotor activity and feeding habits. These high-fat diets also lead to a disruption in a properly functioning clock within metabolic tissues such as liver and adipose [40, 47]. It was also observed that high-fat diets cause a marked disruption in circulating hormone levels such as leptin, corticosterone and TSH in
**Figure 1-2. Core molecular clock components.** BMAL1 and CLOCK regulate the transcription of the PER and CRY family of genes, which in turn inhibits BMAL1 and CLOCK transcriptional activity. Through interactions with RORα, PGC-1α regulates expression of BMAL1 in a rhythmic manner. This activation is inhibited by the nuclear receptor, REV-ERBα (which is induced by BMAL1 and CLOCK), through direct competition with RORα for occupancy on the RORE in the BMAL1 proximal promoter.
humans and rats [40, 63, 64]. Also, evidence points to a loss of circadian control of glucose utilization and insulin action in patients with T2DM, further strengthening the link between metabolic disorders and circadian rhythm [65, 66].

Aside from clinical data linking circadian rhythm and metabolic disease mounting genetic evidence suggests a link as well. This can best be demonstrated by the metabolic phenotypes that develop in clock mutant and knockout mice. ClockΔ mice (C57BL6), that carry a truncated exon 18 as well as a full deletion of exon 19, become hyperphagic (increased food consumption) and obese displaying symptoms of metabolic syndrome including hyperlipidemia, hepatic steatosis and hyperglycemia [67]. In addition, BMAL1 -/- total knockout mice displayed dysfunction in glucose tolerance, insulin sensitivity as well as an increase in total fat mass. Liver specific knock-out of BMAL1 displays a hypoglycemic state during times of fasting suggesting that dysregulation of BMAL1 disrupts glucogeogenesis. This is further supported by the usual circadian rhythmicity of genes involved in gluconeogenesis such as: glucose-6-phosphate translocase (G6PT1), PEPCK, and glucokinase (GCK), being disrupted in BMAL1 -/- knock-out livers [45]. Similar effects are observed when sleeping patterns are affected. Evidence so far collected links short sleep durations with increased BMI as well as a higher incidence in T2DM [68].

1.2.4. PGC-1 Family of Transcriptional Coactivators – Integrating Circadian and Metabolic Inputs
The PGC-1 family is a potent regulator of energy homeostasis and circadian signaling, as briefly stated in Section 1.2.3. Here we describe the family of co-activators, and briefly dissect their role in multiple steps of energy balance and circadian rhythm focusing primarily on liver physiology.

PGC-1α was first identified in brown adipose tissue as an interacting partner with PPARγ (a nuclear receptor first identified in regulating adipogenesis,) [69]. Soon after two other members of the family were identified: peroxisomal proliferator-activated receptor (gamma) coactivator-1 beta (PGC-1β) and PGC-1-related coactivator (PRC) [70-74]. PGC-1α is robustly expressed in highly oxidative tissues such as skeletal muscle, heart, BAT, brain and kidney and to this end has been implicated, along with PGC-1β, in mitochondrial biogenesis [75].

During times of fasting both PGC-1α and PGC-1β aid in coordinating the hepatic fasting response. These co-activators interact with the nuclear receptor PPARα to coordinate fatty-acid oxidation in liver tissue. RNAi mediated knockdown of PGC-1α leads to diminished activation of fatty-acid oxidation genes, as well as decreased rates of fatty-acid oxidation in cultured hepatocytes [76]. Also, PGC-1α but not PGC-1β has been implicated in coordinating the gluconeogenic response in fasting liver tissue through interactions with HNF-4α and FOXO1 [76-79]. Furthermore, PGC-1α itself is induced by administration of dexamethasone (a synthetic glucocorticoid agonist) in vivo - supporting PGC-1α’s role in fasting liver. Ectopic overexpression of PGC-1α in primary hepatocyte cultures is
sufficient to increase mRNA of gluconeogenic genes like PEPCK and G6Pase. Also, PGC-1α null mice show decreased glucose levels in restricted feeding experiments along with decreases in gluconeogenic gene expression [62]. Interestingly, the PGC-1α null mice showed dysregulation of circadian genes suggesting that PGC-1α serves as a potent hub for metabolic and circadian regulation.

1.3. Cellular Role of Protein Ubiquitination

Much of the signaling and regulation in the cell revolves around post-translational modifications of proteins. These modifications include, but are not limited to, phosphorylation, acetylation and ubiquitination. Ubiquitination is a reversible protein modification that can affect protein stability, function and/or localization and has been implicated in multiple cellular processes including: protein degradation, cell cycle regulation, DNA repair and chromatin remodeling [80, 81]. The covalent attachment of ubiquitin is completed in a series of reactions that involves an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and to a target protein via an ubiquitin ligase (E3). Thus, the C-terminus of ubiquitin is covalently attached, in an isopeptide linkage, to the e-amino group of the substrate’s lysine (Lys) residue [80-82]. Target proteins can have one ubiquitin moiety attached, termed mono-ubiquitination, or can have several attached in a chain like structure, called poly-ubiquitination. Poly-ubiquitination is usually a marker for proteasomal degradation, while mono-
ubiquitination has more diverse outcomes [81]. In the human genome there are an estimated 600 E3 ligases, which ensure proper substrate specificity. In opposition of the action of E3 ligases are a super-family of deubiquitinating enzymes, termed DUBs.

1.3.1. Deubiquitinating enzymes (DUBs)

Deubiquitinating enzymes (DUBs) are proteases that catalyze the cleavage of ubiquitin or ubiquitin-like proteins (UBLs) from: ubiquitin-conjugated proteins, ubiquitin precursors, ubiquitin adducts and ubiquitinated remnants from proteasomal mediated degradation. DUBs have three generalized areas of function. First, they aid in the maturation of the ubiquitin monomer from a proprotein state. Second, after a protein has been targeted for degradations DUBs can rescue ubiquitin from targeted proteins contributing to the pool of free ubiquitin. Lastly, DUBs can antagonize the action of E3 ligases by removing ubiquitin from a targeted substrate - this is tantamount to the kinase/phosphatase regulatory system [80, 83]. As with the role of ubiquitination, deubiquitination has been implicated in many cellular pathways including: kinase activation, proteasomal and lysosomal protein degradation as well as transcriptional regulation and DNA repair. The importance of DUB’s in disease models has increased in the last decade, as mutations in DUBs have been linked to cancer as well as neurodegeneration.
There are nearly 100 DUBs encoded by the human genome, categorized into 5 distinct families. Four families encode papain-like cysteine proteases: UCH, USP/UBP, OUT and MJD, while a fifth family encodes a metalloprotease family, JAMM. Herein, we will focus on only the Ubiquitin Specific Protease (USP/UBP) family of deubiquitinases.

1.3.2. **Ubiquitin Specific Protease 2**

Ubiquitin-specific proteases (USP) constitute a major family of deubiquitinases that is emerging as versatile regulators of diverse biological processes including; cell cycle regulation, transcriptional regulation, and mitochondrial dynamics [84-88]. In humans there are predicted to be over 50 USP family members making this the largest class of DUBs. USP’s modulate cellular signaling through deubiquitinating substrate proteins. The catalytic domain of USPs can be broken down into 3 subdomains, which can be likened to the palm, finger and thumb of a hand [80]. The C-terminal of the ubiquitin moiety resides in the groove between the thumb and palm subdomains, while the finger interacts with the globular portion of ubiquitin. Many of the over 50 USP’s in the human genome remain poorly characterized. Herein, we will focus on one USP, USP2, and try to demonstrate a novel function in liver glucose homeostasis.

So far, UPS2 has been characterized as an oncogenic protein shown to interact with the E3 ligases Murine Double Minute 2 (MDM2) and Murine Double Minute X (MDMX), as well as with Fatty Acid Synthase (FAS). Furthermore,
USP2 has been shown to interact with an epithelial sodium channel, ENaC [89]. Deubiquitination of these targets leads to increased stabilization and protein function. USP2 has also been shown to oscillate diurnally in liver tissue and shows decreased oscillation in clock defective mice [90]. However, what effect USP2 has on liver circadian rhythm or gross liver function remains unexplored.

1.4. Prospectus

Over the past decade we have begun to unravel the factors that control circadian rhythm. It has also been well documented the effect disruption of circadian rhythm has on metabolic regulation. Yet, many questions still remain about how hormonal and circadian signals integrate to regulate metabolic processes. USP2 has previously been shown to oscillate in liver tissue, yet USP2’s role in circadian rhythm and liver function remains poorly understood.

Based on these finding we propose the following three aims:

A) Determine the factors involved in USP2 regulation (Chapter 2).

B) Evaluate USP2’s role in liver tissue using gain and loss of function experiments. (Chapter 3).

C) Using cell culture models evaluated the sub-cellular localization of USP2 (Chapter 4).
1.5. References


Chapter 2

Circadian and Nutritional Regulation of USP2-45

2.1. Background

It is estimated that between 3-20% genes oscillate in a rhythmic manner; these are termed circadian output genes and many display tissue specific rhythmicity [1]. Additionally, many of these are regulated by nutritional signals such as in fed and fasting states. Previous work has demonstrated that ubiquitin-specific USP2 shows robust circadian rhythm in liver tissue having a periodicity of ~24hrs. Furthermore, close examination of the gene structure of USP2 reveals that there are two predominant gene products from this locus (Figure 2-1). One gene product is termed USP2-69, which refers to the molecular mass of the protein – 69kDa. There is a second shorter gene product referred to as USP2-45 - having a molecular mass of 45kDa. The two gene products share the last 13 exons composing the C-terminal catalytic domain (Figure 2-1). However, USP2-
69 contains a different 5′ prime 276aa encoding exon while USP2-45 contains a smaller 50aa encoding exon. While previous work has established that these two transcripts are not the result of alternative splicing, but rather two separate gene products being driven by two separate promoters, it is not known whether they are regulated in a similar manner. Herein, we aim to dissect the circadian architecture that regulates USP2 expression.

2.2 Results

2.2.1 USP2-45 is regulated by circadian rhythm

Previous work demonstrated that ubiquitin-specific protease 2 (USP2) oscillates in liver tissue with a periodicity of ~24hrs [2, 3]. The first work profiling this oscillation used probes common to the catalytic domain of both USP2-69 and USP2-45. We hypothesized that since previous published work suggested that both USP2-69 and USP2-45 were not the result of alternative splicing, and under the control of two separate promoters, that their expression might also be differentially regulated [4].

To address this question, we designed two sets of discriminating real-time primers (Figure 2-2A) and measured the mRNA levels in livers of mice throughout a 24hr period of time. First, mice (C57BL/6) were entrained to a 12hr-light/12hr-dark (12:12h LD) cycle with food and water freely available. Next livers were harvested from a group of mice (n=3-5) every four hours for 24hrs and the mRNA levels of USP2-69 and USP2-45 were analyzed. We found that USP2-45
Figure 2.1. Pairwise global alignment of USP2-69 and USP2-45 amino acid sequences. Alignment of USP2-69 and USP2-45 using ClustalW2 software (EMBL-EBI, www.ebi.ac.uk) reveals a conserved catalytic domain in the last 346aa of each protein.
had a more robust circadian oscillation than that of the longer form, USP2-69 (Figure 2-2B, upper left panel). Also, USP2-45 oscillation is not specific to liver tissue as we observe a similar rhythmicity in skeletal muscle (quadriceps) from the same cohort of mice (Figure 2-2B, upper right panel). As a positive control for our circadian liver samples we analyzed the expression of two core clock genes; BMAL1 and REV-ERBα. As expected we observed a robust oscillation of BMAL1 and REV-ERBα in opposite phase of one another in both liver and muscle tissue (Figure 2-2B, lower panels). These data suggest that USP2-45 expression is highest around zeitgeber time ZT10-12 (ZT, ZT0 is the onset of light phase) and reaches its nadir around ZT 4.

We next posed the question whether a properly functioning molecular clock was required for USP2-45 rhythmicity in liver tissue. To address this question we employed the use of a BMAL1 liver specific knock-out (LKO) mouse line. Previous work on this liver specific knock-out demonstrated that lack of liver BMAL1 resulted in a disruption of proper circadian rhythm compared to wild-type controls [5]. We proceeded as describe above; mice were kept on a 12:12 LD cycle and mice were sacrificed (n=3-5) every 4 hours for a period of 24hrs. As expected there is no detection of Bmal1 in liver specific knock-out mice (Figure 2-3, upper left panel) Interestingly though, when BMAL1 is absent we see a complete disruption of USP2-45 rhythmicity (Figure 2-3, upper-right panel). We do observe increased expression of USP2-45 in BMAL1 LKO mice at the nadir of USP2-45 expression at time points ZT 1 and ZT 22. Except for ZT 4 all other
Figure 2-2. USP2-45 demonstrates diurnal oscillation in liver and muscle tissue. (A) Diagram of USP2 gene structure and the position of the real-time primers used to discriminate between USP2-69 and USP2-45. (B) Circadian oscillation of USP2-69 and USP2-45 in liver and muscle tissue as well as Bmal1 and Rev-Erbα (n=3-5). Bmal1, brain and muscle ARNT-like 1; Rev-Erbα. Data represent mean ± s.em. * p < 0.05.
time points are significantly lower compared to BMAL1 wild-type mice. In addition, PEPCK and E4BP4 (Nfil3, a transcriptional repressor) are also rhythmically disrupted in BMAL1 LKO mice. It is interesting to point out that the two time points (ZT 1 and ZT 22) where USP2-45 expression is higher in BMAL1 LKO mice E4BP4 levels are significantly lower. We will address this observation in more depth, later in this chapter. From these studies we conclude that a properly functioning clock is required for proper USP2-45 expression. Whether BMAL1 is a direct regulator of USP2-45 rhythmicity remains unclear.

At this point it must be noted that we have been unable to detect endogenous USP2-45 protein consistently due to a lack of specific antibodies that react with mouse USP2-45. We have tried several commercially available antibodies but so far without any success. We will address this shortcoming in the “Future Directions” section of this chapter.

2.2.2. Tissue Distribution

We also looked at the tissue distribution of USP2-69 and USP-45 (Figure 2-4). We again used our discriminating real-time primer sets and looked at brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle (quadriceps), heart, brain, spleen, kidney and liver. While we cannot quantify the difference of USP2-45 relative to USP2-69 in different tissues, because of potential differences in primer efficiencies, we can get an idea of how widely expressed
Figure 2-3. USP2-45 circadian oscillation is disrupted in liver specific BMAL1 knock-out livers. One-day temporal expression profile of selected circadian and metabolic genes in liver tissue from Bmal1 WT mice (black circle) and Bmal1 liver specific knock-out (LKO) mice (white circle). Shown is the mean and SEM of a pooled set of cDNA (n = 4, for each time point) from each time point run in triplicate (some error bars are too small to see on this scale). BMAL1, Brain and muscle ARNT-like1; USP-45, Ubiquitin-specific protease 2-45; PEPCK, phosphoenolpyruvate carboxykinase 1; E4BP4, E4 promoter binding protein 4. * p < 0.05.
**Figure 2-4. Tissue distribution of USP2-69 & USP2-45**. Shown is the qPCR profile of USP2-45 (white bars) and USP2-69 (black bars) in brown adipose tissue (BAT), white adipose tissue (WAT), muscle (quad), heart, brain, spleen, kidney and liver tissues. cDNA was pooled from 2 individual male (C57BL6J) mice and run in a triplicate.
each gene product is. USP2-45 seems to be widely expressed in most tissues except splenic tissue. USP2-69 is also similarly distributed amongst tissues analyzed. USP2-45 seems more highly expressed in skeletal muscle, heart, brain and kidney than in liver, BAT and WAT. On the other hand USP2-69 shows higher expression in BAT and WAT than in skeletal muscle, heart and brain tissue. The physiological relevance of the differences in tissue distribution still remains unclear.

2.2.3. Nutritional regulation of USP2-45 and USP2-69

Many genes involved in energy metabolism (e.g. fatty acid oxidation, lipogenesis, gluconeogenesis) oscillate in a circadian manner due to the rhythmic nature of feeding cycles (e.g. fasting/feeding). These feeding cycles also cause oscillations in hormones like insulin and glucagon as well as stress hormones such as cortisone (or corticosterone in mice). Since the peak of USP2-45 expression occurs around the light-dark transition corresponding to fasting or semi-fasting state we hypothesized that USP2-45 might also be regulated by nutritional inputs. To test our hypothesis we again used our 2 sets of discriminating real-time primers to elucidate whether USP2-45 or USP2-69 responds to nutritional signals.

We found that USP2-45 mRNA expression is significantly induced in response to starvation and reduced following overnight refeeding (Figure 2-5A). As shown in Figure 2-5A, mRNA expression of USP2-45 is induced by
approximately 3.2-fold in the liver during fasting. In contrast, mRNA levels of USP2-69 remain similar under these feeding conditions. The induction of USP2-45 by starvation appears to be liver-specific, as mRNA levels for both isoforms remain largely unchanged in white adipose tissue and skeletal muscle. Since we observed a decrease in USP2-45 mRNA levels during refeeding this may suggest a role for insulin in repressing the expression of USP2-45. To test this possibility, we injected mice with streptozotocin (STZ) to destroy β-cell function in the pancreas - disrupting insulin signaling. When we injected mice with STZ we observed a dramatic increase in the mRNA level of USP2-45 in liver tissue compared to saline control mice. The mRNA levels of USP2-69 remain unchanged. This suggests insulin plays a repressive role in USP2-45 regulation in vivo.

To determine the nature of the starvation signals that regulate USP2-45 expression, we treated cultured mouse primary hepatocytes with glucagon, hydrocortisone, an endogenous glucocorticoid in rodents, and insulin alone or in combination. As expected, the combination of glucagon and hydrocortisone strongly induces the expression of PEPCK and IGFBP1, known glucocorticoid targets in the liver (Figure 2-6, right, upper and lower panel). USP2-45 expression is robustly induced by hydrocortisone, which is further augmented in the presence of glucagon. In contrast, USP2-69 mRNA levels remain largely unaffected by these treatments (Figure 2-6, left, upper and lower panel). Similar to PEPCK and IGFBP1, the induction of USP2-45 by hydrocortisone and glucagon is strongly suppressed by insulin treatments. These results indicate
Figure 2-5. Nutritional regulation of USP2 isoforms. (A) Realtime qPCR analysis of USP2-45 and USP2-69 mRNA in liver, white adipose tissue (WAT), and skeletal muscle from fed (open), fasted (filled), and refed (gray) mice. Shown below is a schematic of the USP2 gene locus and the qPCR primers (arrowheads) used to detect USP2-45 and USP2-69 isoforms. Data represent mean ± s.e.m (n=4). (B) Realtime qPCR analysis of USP2-45 and USP2-69 mRNA in liver treated with saline (open) or STZ (filled). Data represent mean ± s.e.m (n=3). * p < 0.05.
Figure 2-6. Hormonal control of USP2 isoforms. qPCR analysis of total RNA from primary hepatocytes treated with hydrocortisone, glucagon and insulin (6hr treatment). Data represent mean ± stdev of samples from one representative experiment assayed in triplicate. * p < 0.01, hydrocortisone plus glucagon vs. control. # p<0.01 insulin vs. no insulin.
that USP2-45 is regulated by nutritional signals (e.g. glucagon and insulin) and is a target of hepatic glucocorticoid signaling.

2.2.4. PGC-1 family of co-activators’ role in USP2-45 regulation

Previous work done on the PGC-1 family of co-activators has suggested a role for coordinating circadian and metabolic signaling [6]. PGC-1α has a role in regulating core circadian clock genes such as BMAL1 and REV-ERBα as well as gluconeogenic genes like PEPCK and G6Pase. PGC-1α itself is regulated by circadian rhythm as well as being induced during times of food deprivation in liver tissue controlling gluconeogenesis in the liver [7, 8]. We previously identified USP2 as a target of PGC-1α using wild-type primary hepatocyte microarrays in gain-of-function experiments. Using our USP2 discriminating primers we observed roughly a 35-fold and 30-fold induction of USP2-45 in primary hepatocytes transduced with either adenoviral PGC-1α or PGC-1β (another PGC-1 family member), respectively (Figure 2-7A). Over expression of PGC-1α or PGC-1β had no significant effect on USP2-69 mRNA levels. ERRα and SOD2, two known targets of PGC-1α and PGC-1β were also significantly elevated. All results were normalized to cells transduced with adenoviral GFP. Similar results were obtained with C2C12 myotubes transduced with either PGC-1α or PGC-1β (Figure 2-7B). The relative induction of USP2-45 in primary hepatocytes is higher than that of C2C12 myotubes. We attribute this to virus infectivity of primary hepatocytes compared to C2C12 myotubes and not elevated base-line
levels of USP2-45 in C2C12 cells. We next sought to elucidate whether this regulation was physiologically relevant in vivo. To test this we transduced mouse livers with either adenoviral GFP, PGC-1α (Figure 2-7C), or PGC-1β (Figure 2-7D). In both cases we observed a significant increase in USP2-45 mRNA levels. Interestingly, we observed a significant decrease in USP2-69 levels in both PGC-1α and PGC-1β transduced livers. Again, ERRα and SOD2 were also significantly induced suggesting viral transduction of the liver worked. Taken together this work suggests that the PGC-1 family of co-activators is sufficient to drive induction of USP2-45, specifically. However, the PGC-1 family does not contain a DNA binding domain and therefore must interact with other DNA-binding factors to regulate transcription [9]. Because of this we next wanted to identify transcription factors that facilitated PGC-1’s regulation of USP2-45.

2.2.5. Factors involved in USP2-45 regulation

To identify factors involved in the regulation of USP2-45, we cloned out a 3.7kb fragment of the proximal promoter. We then used this construct to perform promoter reporter luciferase based assays in BOSC cells. For these experiments, we will be using luciferase enzyme activity as a marker for promoter activity. We initially narrowed our list of transcription factors to a handful of known PGC-1 interacting partners (both from published and unpublished (lab data) sources) [9]. From this screen we identified hepatic nuclear factor 4-alpha (HNF-4α) and estrogen-related receptor gamma (ERRγ) as potent regulators of the USP2-45
Figure 2-7. Over-expression of PGC-1α or PGC-1β is sufficient to induce Usp2-45 Expression. PGC-1α and PGC-1β are over-expressed in (A) 1° hepatocytes and (B) C2C12 myotubes using an adenoviral delivery system. Data represent mean ± stdev of samples from one representative experiment assayed in triplicate. Tail-vein injection was used to deliver PGC-1α (C) and PGC-1β (D) adenovirus to liver tissue in mice. For panel C, n = 6 for both GFP and PGC-1α. For panel D, n = 4 for GFP and n = 5 for PGC-1β. Data represent mean ± s.e.m. Ubiquitin-specific protease 2-69 (USP2-69), Ubiquitin-specific protease 2-45 (USP2-45), Estrogen related receptor alpha (ERRα), Superoxide dismutase 2 (SOD2). * p < 0.05.
In both instances HNF-4α and ERRγ were able to synergize with PGC-1α or PGC-1β on the USP2-45 promoter to drive increases in luciferase expression (Figure 2-8A&B). In fact, over-expression of HNF-4α or ERRγ alone was sufficient to significantly increase promoter activity. Other factors included in the screen are shown in Figure 2-8C. We saw a significant increase in USP2-45 promoter activity when both thyroid receptors alpha and beta (TRα/TRβ) were cotransfected, separately, with PGC-1α. However, SREBP-1c, RORα, FOXA2 and HCFC1 had little effect on promoter activity alone or coexpressed with PGC-1α. Interestingly, over-expression of GR was unable to induce USP2-45 promoter activity (Figure 2-8D). This was a little counter-intuitive and we thought that maybe our GR construct was non-functional. To verify the functionality of our GR construct we used a synthetic promoter containing a glucocorticoid response element, termed TAT-GRE-luc. In these experiments GR was able to induce luciferase expression in both the absence and presence of ligand (DEX) (Figure 2-8E). This may suggest that the GRE responsible for regulating USP2-45 expression resides outside of the 3.7kb proximal promoter.

Through the use of this small-scale promoter reporter screen we were able to identify two potent regulators of USP2-45, HNF-4α and ERRγ. We next tried to elucidate what parts of the USP2-45 promoter were critical for this regulation. To this end we cloned two smaller (0.6k and 1.7kb) fragments of the
**Figure 2-8.** HNF-4α and ERRγ synergize with PGC-1α and PGC-1β on the 3.7kb USP2-45 promoter. Using a luciferase promoter construct containing the 3.7kb upstream promoter of USP2-45 demonstrates that PGC-1α and PGC-1β can synergize with (A) HNF-4α and (B) ERRγ to increase promoter activity. HNF-4α, hepatic nuclear factor 4 (alpha); ERRγ, estrogen related receptor (gamma). (C) Small-scale screen of other known PGC-1α interaction partners; TRα, thyroid hormone receptor (alpha); TRβ, thyroid hormone receptor (beta); SREBP-1c, sterol regulatory element-binding protein-1c; GR, glucocorticoid receptor; FOXA2, forkhead box A2; HCFC1, host cell factor C1, RORα, RAR-related orphan receptor (alpha). * p < 0.05 (D) Transfection of GR in combination with 3.7kb USP2-45 promoter plus or minus dexamethasone (100nM, 6hrs). (E) Transfection of GR in combination with TAT-GRE promoter with or without 100nM dexamethasone for 6hrs. * p < 0.05. # p < 0.05 GR v. GR + DEX. Data represent mean of two wells ± stdev.
USP2-45 promoter (USP2-45-luc). In both the 0.6kb and 1.7kb fragments, ERR\(_\gamma\) was able to synergize with PGC-1\(\alpha\) or PGC-1\(\beta\) to increase luciferase expression (Figure 2-9A&B). However, it does appear the synergy is more robust in the 1.7kb fragment. A similar conclusion can be made when looking at HNF-4\(\alpha\). In both fragments HNF-4\(\alpha\) is able to synergize with PGC-1\(\alpha\) or PGC-1\(\beta\) to increase luciferase expression. But again this synergy is more robust in the 1.7kb fragment. Direct comparison between the two fragments is difficult since their baseline activity levels are different. In these experiments we transfected a constant amount of plasmid instead of trying to match activity levels. Another approach would be to titrate in the reporters until a similar baseline is reached and then analyze transcriptional regulation. Regardless, in these experiments it does seem as though the 0.6kb fragment is sufficient to increase promoter activity. This maybe due to the 0.6kb fragment containing the bare minimum of elements required for proper activation, while the 1.7kb fragment contains more regulatory elements - further augmenting the induction. However, we still have to identify binding sites for either ERR\(_\gamma\) or HNF-4\(\alpha\). And the requirement of either HNF4\(-\alpha\) or ERR\(_\gamma\) for proper USP2-45 regulation still remains to be elucidated.

So far we have identified transcription factors responsible for the positive regulation of USP2-45. We next wanted to explore what transcription factors were involved in the negative regulatory arm of USP2-45. We focused on four prominent repressors of circadian rhythm: small heterodimer partner (SHP,
Figure 2-9. The 0.6kb region of the USP2-45 promoter is sufficient for PGC-1α and PGC-1β regulation via interactions with HNF-4α and ERRγ. Reporter gene assays using (A) 0.6kb and (B) 1.7kb promoters of USP2-45 in the presence of ERRγ, PGC-1α and PGC-1β. Reporter gene assays using (C) 0.6kb and (D) 1.7kb promoters of USP2-45 in the presence of HNF-4α, PGC-1α and PGC-1β. Data represents mean of two wells ± stdev. * p < 0.05.
NROB2), REV-ERBα (Nr1d1), REV-ERBβ (Nr1d2) and E4 promoter-binding protein 4 (E4BP4, Nfil3). We co-transfected BOSC cells with PGC-1α and HNF-4α (plus 3.7kb USP2-45-luc), and a titrating dose of one of the four repressors. In this instance E4BP4 was able to antagonize the stimulatory effect of HNF-4α and PGC-1α in a dose dependent manner (Figure 2-10A). Both SHP and REV-ERBα had little to no effect on the HNF-4α/PGC-1α activation, while REV-ERBβ repressed activation at only a higher dose. Furthermore, E4BP4 was able to repress HNF-4α/PGC-1α activation in both the 0.6kb and 1.7kb fragments (Figure 2-10B). In section 2.2.2, we observed elevated levels of USP2-45 at ZT 4 and 22 in BMAL1 LKO animals compared to wild-type controls. This seems to correspond to a decrease in E4BP4 mRNA levels in LKO samples (Figure 2-2, lower right panel). It has also been demonstrated that E4BP4 plays a significant role in the repressive arm of insulin signaling [10]. This is still mostly circumstantial evidence, however, taken together with our promoter reporter experiments, E4BP4 may play a major role in the circadian and nutritional regulation of USP2-45.

2.3. Conclusions

1.) The USP2-45 gene product is regulated by both circadian and nutritional inputs.

2.) Members of the PGC-1 family of transcription factors are sufficient to induce
USP2-45 expression - both *in vitro* and *in vivo*.

3.) USP2-45 regulation is mediated in part by HNF-4α or ERRγ and is antagonized by E4BP4.

2.4. Discussion

Our studies indicate that USP2-45 was the main isoform of USP2 oscillating in both liver and muscle tissue and that knock-out of BMAL1, in liver tissue, caused complete loss of this rhythm. The precise molecular basis for USP2-45 regulation still remains poorly understood. However, we have (through reporter gene assays) identified the PGC-1 family of co-activators as potent activators of USP2-45. Both PGC-1α and PGC-1β are rhythmically expressed in the liver and PGC-1α regulates core clock genes, including BMAL1 and REV-ERBα [6]. We also demonstrated that this activation is potentially mediated by the nuclear receptors, HNF-4α and ERRγ, and that this activation was antagonized by the transcriptional repressor, E4BP4.

Along with circadian inputs, USP2-45 is also regulated by fasting signals in the liver. In cultured hepatocytes, fasting hormones, hydrocortisone and glucagon potently activated, while insulin repressed, USP2-45 expression (Figure 2-11). Given that PGC-1α strongly induces USP2-45 expression, it is possible that USP2-45 is a component of the hepatic metabolic response orchestrated by PGC-1α during fasting and throughout light/dark cycles. In this
Figure 2-10. E4BP4 antagonizes the action of HNF-4α and PGC-1α on the promoter of USP2-45. (A) Reporter gene assays using 3.7kb USP2-45-luc in the presence of SHP, REV-ERBa, REVERBa or hE4BP4. Reporter gene assays using (B) 0.6kb or (C) 1.7kb USP2-45-luc in the presence of hE4BP4. Data represents mean of two wells ± stdev. * p < 0.05; promoter alone v. HNF-4α/PGC-1α. # p < 0.05; HNF-4α/PGC-1α v. HNF-4α/PGC-1α + hE4BP4.
Figure 2-11. Both nutritional and circadian inputs regulate USP2-45 expression. Members of the PGC-1 transcription co-activator family potently activate USP2-45 expression while E4BP4 represses USP2-45 expression. Fasting hormones, hydrocortisone and glucagon, induce USP2-45 expression while insulin reduces this induction.
regard USP2-45 may be a direct target of GR, which itself is a transcriptional partner for PGC-1α. Alternatively, PGC-1α may regulate USP2-45 gene expression indirectly through its modulation of the clock pathway. However, more work is needed to dissect these emerging regulatory pathways.

2.5. Future Directions

2.5.1 USP2-45 protein detection

As discussed previously, we were unable to detect protein levels for USP2-45. This is important for validation of our observations regarding regulation of USP2-45 mRNA levels. Since we have tried several antibodies against USP2-45 with little success a more aggressive approach might be required. One possibility would be to construct a transgenic line of mice containing a HA/Flag cassette at the end of the 13th exon of USP2-45 (also would be present in USP2-69). Alternatively, the cassette could be placed at the 5’ end of exon 1 of USP2-45. This would lend specificity, as it would not be present in the USP2-69 isoform. This would be similar to a transgenic construct but the endogenous regulatory elements would be intact.

2.6 Materials and Methods

*Cultured primary hepatocytes*. Primary hepatocytes were isolated from female C57/Bl6J mice using collagenase type-II (Invitrogen), as previously described
Hepatocytes were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% bovine growth serum and antibiotics at 37°C and 5% CO₂. Cells were switched to DMEM supplemented with 0.1% BSA for 16-24 hrs before treatments with hydrocortisone (1µM), glucagon (40 nM) or insulin (100 nM) for 6 hrs. For adenoviral transduction, recombinant adenoviruses were generated using AdEasy adenoviral vector (Stratagene) as previously described [12]. Hepatocytes were transduced for 48 hrs at similar moiety of infection before RNA isolation and gene expression analysis.

**Gene expression analysis.** Total hepatocyte RNA was isolated using Trizol (Invitrogen), reversed transcribed using MMLV reverse transcriptase, and analyzed by quantitative PCR using Sybr Green method. The primers used for qPCR analysis were described in previous studies [6, 12].

**In vivo mouse studies.** C57BL/6J mice were kept on a 12:12 light-dark cycle with food and water freely available. For fasting/refeeding studies, mice were provided food ad lib, fasted for 20 hrs, or refed for 18 hrs following fasting. Tissues were harvested at the same time and frozen, using dry ice, and subsequently prepped for gene expression analysis. Bmal1 flox/flox mice were obtained from the Jackson Laboratory. Liver-specific Bmal1 knockout mice were generated by breeding the flox/flox mice with Albumin-cre transgenic mice. Tissues were harvested every 3 hrs for a period of 24 hrs for gene expression
studies. The University Committee on Use and Care of Animals (UCUCA) at the University of Michigan approved all animal procedures performed in this paper.

**Luciferase Assay.** BOSC cells were transiently transfected using either Lipofectamine/ Plus Reagent (Invitrogen) or polyethyleneimine (Polysciences, Inc.) with desired plasmids. USP2-45 promoter constructs were cloned from liver genomic DNA and sub-cloned into the pGL-Basic luciferase vector (Promega). 25-50ng of reporter was used in each well and all transfection reagents contained the same amount of plasmid DNA using pCDNA as an empty vector control [6]. Cells were transfected in serum free DMEM, supplemented with antibiotics over-night in 24-well plate and 1ml of DMEM, supplemented with 10% BGS and antibiotics was added the following day. Cells were harvested 48 hours post-transfection and cell lysates were prepared using the BD Moonlight™ luciferase assay system (BD Bioscience) and read using a Molecular Devices LMax luminometer.
Notes

Portions of this work are included in a paper in preparation entitled: “Ubiquitin-specific protease 2 regulates hepatic gluconeogenesis and diurnal glucose metabolism through 11β-hydroxysteroid dehydrogenase 1,” Matthew M. Molusky, Siming Li, Di Ma, Lei Yu, and Jiandie D. Lin.

M. M. Molusky and J. D. Lin designed the experiments and prepared the manuscripts. D. Ma was responsible for BMAL1 liver specific knock-out samples shown in Figure 2-3.
2.7 References

Chapter 3

USP2-45 Regulates Hepatic Glucose Homeostasis

3.1. Background

Hepatic gluconeogenesis is important for maintaining blood glucose homeostasis in mammals during prolonged fasting. Glucocorticoids and glucagon are major physiological hormones that stimulate the expression of gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), whereas insulin suppresses the action of these counter regulatory hormones in the liver [1-3].

Recent studies have demonstrated that circadian clock exerts profound influences on energy metabolism and is required for maintaining energy and nutrient homeostasis [4-10]. Disruption of pancreatic clock in mice impairs β-cell
function and leads to hypoinsulinemia and diabetes [11, 12], whereas ablation of liver clock results in hypoglycemia following starvation and at certain time points during the day due to impaired hepatic gluconeogenesis [13, 14]. In addition, clock protein cryptochrome (CRY) modulates hepatic cAMP signalling and gluconeogenesis [15]. As such, hormonal and circadian signals likely converge on key regulatory nodes to coordinate hepatic gluconeogenesis and glucose secretion.

Reversible protein ubiquitination and deubiquination modulate the biochemical functions of target proteins. The latter is carried out by deubiquitinating enzymes, which remove ubiquitin or ubiquitin-like protein from their substrates [16, 17]. Ubiquitin-specific proteases (USP) constitute a major family of deubiquitinases that is emerging as versatile regulators of diverse biological processes, including cell cycle regulation, signalling, transcriptional regulation, mitochondrial dynamics [18-22]. Whether USP members are nutritionally regulated and participate in the regulation of glucose metabolism remain unexplored. Herein, we describe the first work done characterizing ubiquitin specific protease 2 as a factor controlling hepatic glucose output via liver specific increases in glucocorticoid signalling.

3.2 Results

3.2.1 USP2-45 stimulates hepatic gluconeogenesis and glucose output.
De novo glucose synthesis via gluconeogenesis is essential for maintaining blood glucose levels during starvation in mammals. To determine whether USP2-45 regulates hepatic glucose production, we transduced chow-fed mice via tail vein injection of recombinant adenoviruses expressing GFP (control) or Flag/HA-tagged USP2-45. Compared to control, adenoviral-mediated expression of USP2-45 in the liver results in elevated plasma glucose and insulin concentrations (Figure 3-1A). We next performed pyruvate tolerance test (PTT) to directly assess hepatic gluconeogenic function in transduced mice. We inject intraperitoneally (i.p.) a single bolus of pyruvate, a gluconeogenic substrate, and measured blood glucose levels at different time points. Mice transduced with USP2-45 adenovirus have significantly higher blood glucose levels following pyruvate injection (Figure 3-1B), suggesting that hepatic gluconeogenic activity is augmented by USP2-45. Consistently, we found that mRNA expression of PEPCK is increased by USP2-45 (Figure 3-1C). The expression of G6Pase remains unchanged.

We next examined whether USP2 is required for maintaining normal blood glucose levels during starvation. We generated recombinant adenoviruses that express shRNA directed toward USP2 (Figure 3-2A) and transduced C57BL/6J mice with control or siUSP2 adenoviruses. Measurements of fasting blood glucose indicate that mice with hepatic knockdown of USP2 have significantly lower glucose levels (Figure 3-2B). In addition, circulating insulin concentration is also reduced in the knockdown group, although the difference only reaches borderline statistical significance (p=0.07). Consistently, we found that mice
transduced with siUSP2 adenovirus have impaired ability to convert pyruvate into glucose during PTT (Figure 3-2C), suggesting that hepatic gluconeogenesis and glucose output are impaired by RNAi knockdown of USP2 in the liver. Gene expression analysis revealed that PEPCK mRNA expression is lower in mice transduced with siUSP2 adenovirus (Figure 3-2D). We conclude from these studies that USP2-45 regulates hepatic gluconeogenesis and is required for plasma glucose homeostasis.

3.2.2. Diurnal regulation of glucose homeostasis by USP2

In mammals, the biological clock regulates diverse behavioral and physiological rhythms, notably pathways of nutrient and energy metabolism [4, 23-25]. To explore the role of USP2-45 in circadian glucose regulation, we transduced C57BL/6J mice with control or siUSP2 (construct #1) adenoviruses and measured plasma glucose levels under restricted feeding conditions. Restricted feeding has been demonstrated to play a dominant role in setting the phase of clock and metabolic gene expression in peripheral tissues [5, 26]. We observed significantly lower plasma glucose levels at zeitgeber time (ZT, ZT0 is the onset of light phase) 6, 12, and 24, but not ZT18, when mice were fed exclusively during dark phase (Figure 3-3A). Following a switch to daytime feeding, siUSP2 transduced mice have significantly lower glucose levels at ZT24. Gene expression analysis at ZT0 and ZT12 reveals that mRNA levels of USP2, PEPCK and glucose-6-phosphate transporter (G6PT), but not G6Pase, are
Figure 3-1. USP2-45 promotes hepatic gluconeogenesis. (A) Plasma glucose and insulin concentrations in transduced mice measured under fed condition. (B) Pyruvate tolerance test in mice transduced with GFP (open squares, n=7) or USP2-45 (filled triangles, n=7) adenovirus. (C) qPCR analysis of PEPCK and G6Pase gene expression in livers from mice transduced with GFP (open) or USP2-45 (filled) adenovirus. Data in A-C represents mean ± s.e.m. * p < 0.05, GFP vs. USP2-45.
Figure 3-2. Knock-down of USP2 in chow fed mice results in hypoglycaemia and decreased liver glucose production. (A) BOSC cells were co-transfected with Flag-HA tagged USP2-45 and either siUSP2 construct #1 or #2. Cell lysates were blotted for HA (upper row) to detect USP2-45 knock-down; protein loading was controlled for by blotting for tubulin (lower). (B) Blood glucose and insulin levels in mice transduced with control or siUPS2 adenovirus following overnight fast. (C) Pyruvate tolerance test in mice transduced with control (filled squares, n=6) or siUSP2 (open triangles, n=7) adenovirus. (D) qPCR analysis of liver gene expression from mice transduced with control (filled) or siUSP (open) adenovirus. Data in D-F represents mean ± s.e.m. * p < 0.05, control vs. siUSP2.
Figure 3-3. USP2-45 regulates circadian glucose metabolism. (A) Blood glucose levels in mice transduced with control (red, n=11) or siUSP2 (blue, n=12) adenovirus with feeding restricted to night-time (upper panel) or three days after a switch to day-time feeding (lower panel). Data represents double plotted mean ± s.e.m. (B) qPCR analysis of liver gene expression from restricted day-time fed mice transduced with control (filled) or siUSP2 (open) harvested at ZT0 or ZT12. Data represents mean ± stdev using pooled RNA assayed in triplicate. * p < 0.05, control vs. siUSP2.
reduced in response to RNAi knockdown of USP2 (Figure 3-3B). We conclude from these experiments that not only is USP2-45 a target of circadian signaling (Chapter 2) in the liver it also participates in diurnal regulation of glucose metabolism.

3.2.3. Hepatic overexpression of USP2 exacerbates glucose intolerance in diet-induced obese mice

Hepatic gluconeogenesis and glucose secretion are elevated in diabetes and exacerbate hyperglycemia in diabetic states. We next examined whether USP2 expression is altered in the liver of high-fat diet fed (HFD) obese mice. Compared to lean control, mRNA expression of Usp2-45 is significantly decreased in the liver of diet-induced obese mice (Figure 3-4A). Chronic high-fat feeding results in obesity and hyperinsulineamia. Given that USP2-45 expression is suppressed by insulin in hepatocytes (Chapter 2), our results suggest that insulin may inhibit hepatic USP2-45 expression in obese mice, potentially serving as an adaptive mechanism to restrain hepatic gluconeogenesis when blood glucose is readily available and insulin levels are high.

We next examined whether liver-specific overexpression of USP2 exacerbates glucose intolerance in insulin resistant state. We transduced HFD-fed mice with recombinant adenoviruses expressing GFP or USP2-45. Compared to control, blood glucose concentrations are significantly elevated in mice transduced with USP2-45 adenovirus under fed and fasted conditions.
(Figure 3-4B). Fasting blood insulin concentrations are also significantly higher in mice transduced with USP2-adenovirus (Figure 3-4C). To rule out the possibility that enhanced glycogenolysis may contribute to elevated blood glucose in response to USP2-45; we measured liver glycogen content in transduced mice. Compared to GFP, USP2-45 significantly increases liver glycogen content (Figure 3-4D&E). In contrast, hepatic triglyceride content is modestly affected by USP2-45. Because gluconeogenesis is important for the indirect pathway of hepatic glycogen synthesis [27], these results suggest that USP2-45 augments gluconeogenic flux that leads to increased glucose production and glycogen storage. Glucose tolerance test (GTT) indicates that adenoviral-mediated overexpression of USP2-45 further impairs glucose tolerance in transduced mice (Figure 3-4F). Consistently, blood glucose levels remain significantly elevated at all time points in an insulin tolerance test (ITT) (Figure 3-4G).

Analysis of serum samples also revealed a significant decrease in free fatty acid concentrations in USP2-45 mice in both fed and fasting conditions. Ketone bodies (fasting only) and serum triglycerides, however, remained similar in both groups (Table 3-1 & 3-2). Interestingly, while body weights remain similar between both groups the liver weight to body weight ratios in USP2-45 mice were significantly smaller compared to GFP control mice (Table 3-3). One explanation for this could be changes in the level of macronutrients, like glycogen and triglycerides. However, we observe no change in liver triglycerides, and
Figure 3-4. Hepatic USP2-45 overexpression promotes glucose intolerance in high-fat fed mice. (A) qPCR analysis of hepatic USP2 gene expression in mice fed chow (filled) or high-fat diet (open). Data represents mean ± s.e.m (n=3). (B) Plasma glucose concentrations in HFD-fed mice transduced with GFP (open, n=7) or USP2-45 (filled, n=7) adenovirus. (C) Plasma insulin concentrations in ad lib transduced mice. (D) Liver glycogen and triglyceride content in transduced mice. (E) PAS stain of paraffin-embedded liver sections. (F) Glucose tolerance test in HFD-fed mice transduced with GFP (open diamonds, n=8) or USP2-45 (filled squares, n=8) adenovirus. (G) Insulin tolerance test in transduced mice. For F&G data represents mean ± s.e.m. * p < 0.05, GFP vs. USP2-45.
Tables 3.1 – 3.3. Serum metabolites and other physiological characteristics of mice transduced with either GFP or UPS2-45 adenovirus. Table 3.1 Serum free fatty acid, ketone body, and cholesterol levels in fed and fasting states. Table 3.2 Fasting serum triglyceride. Table 3.3 Fed and fasting body weights, liver weight ratios (LW/BW), and white adipose tissue weight ratios (WATW/BW) at time of harvest. Liver and white adipose weights are normalized to body weights. Data represents mean ± s.e.m. Fed group n = 8; fasted group n = 7. * p < 0.05 GFP v. UPS2-45.

Table 3.1: Serum Free Fatty-Acids [mM] Ketone Bodies [nM] Serum Cholesterol [md/dL]

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<td>GFP</td>
<td>0.22 ± 0.011</td>
<td>0.613 ± 0.04</td>
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<td>0.734 ± 0.083</td>
<td>88.38 ± 2.3</td>
<td>97.07 ± 4.4</td>
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<td>USP2-45</td>
<td>0.18 ± 0.01*</td>
<td>0.46 ± 0.024*</td>
<td>---</td>
<td>0.648 ± 0.052</td>
<td>80.86 ± 2.2*</td>
<td>80.31 ± 2.9*</td>
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Table 3.2: Free Glycerol [mg/dL] Total [mg/dL] Serum Triglycerides [mg/dL]

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<tr>
<td>GFP</td>
<td>45.77 ± 2.2</td>
<td>54.71 ± 4.0</td>
<td>68.31 ± 3.6</td>
<td>101.26 ± 8.9</td>
<td>22.53 ± 3.0</td>
<td>46.55 ± 6.1</td>
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<tr>
<td>USP2-45</td>
<td>40.65 ± 1.6</td>
<td>58.25 ± 4.6</td>
<td>65.92 ± 2.4</td>
<td>103.45 ± 10.8</td>
<td>25.28 ± 2.8</td>
<td>45.20 ± 7.9</td>
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Table 3.3: Body Weight (g) LW/BW (%) WATW/BW (%)

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<tr>
<td>GFP</td>
<td>35.1 ± 0.71</td>
<td>29.23 ±</td>
<td>4.89 ± 0.14</td>
<td>4.81 ± 0.41</td>
<td>4.11 ± 0.27</td>
<td>4.21 ± 1.2</td>
</tr>
<tr>
<td>USP2-45</td>
<td>35.73 ± 0.69</td>
<td>30.20 ±</td>
<td>4.10 ± 0.41*</td>
<td>3.76 ± 0.24*</td>
<td>4.19 ± 0.32</td>
<td>5.24 ± 0.69</td>
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interestingly, an increase in glycogen content in USP2-45 livers. As of yet this phenotype has yet to be explained.

Analysis of hepatic gene expression indicates that PEPCK mRNA expression is significantly induced by USP2-45 (Figure 3-5A). Unlike chow-fed mice, the mRNA levels of G6Pase and G6PT1 are also increased in response to USP2-45 overexpression. Excess glucocorticoid signaling is responsible for the development of diabetes in patients with Cushing’s syndrome and has also been implicated in the pathogenesis of metabolic syndrome [28-31]. In humans, cortisone is released by the adrenal gland and converted to active cortisol locally in tissues by HSD1. A major physiological target of glucocorticoid signaling in the liver is gluconeogenesis; as such we postulated that USP2-45 might augment hepatic glucocorticoid signaling. In support of this, we found that mRNA levels for several known glucocorticoid target genes, including IGFBP1, TSC22D3, DUSP1, and ANGPTL4 [32], are also induced by USP2-45 in the liver. While mRNA levels of CCL2, IL6, and TNFα, genes involved in inflammatory response, remain similar between two groups, the expression of several lipogenic genes, including fatty acid synthase (FAS), acetyl-CoA carboxylase 2 (ACC2), and glucokinase (GCK), appears to be enhanced by USP2-45 (Figure 3-5A). Interestingly, both mRNA and protein levels of HSD1 are significantly increased in response to hepatic overexpression of USP2-45 (Figure 3-5B). Increased HSD1 protein expression was confirmed using ImageJ software analysis (Figure 3-5C). In contrast, the expression of Sec8, another ER membrane protein, and GR remains similar. These results are consistent with elevated plasma insulin
Figure 3-5. Overexpression of USP2-45 in liver tissue increases expression of glucocorticoid responsive genes. (A) qPCR analysis of liver gene expression in mice transduced with GFP (open) or USP2-45 (filled) adenovirus. Data represents mean ± s.e.m. * p < 0.05, GFP vs. USP2-45. (B) Immunoblotting analysis of total liver lysates from transduced mice as indicated. (C) ImageJ quantitation of HSD1 protein levels in panel B normalized to Sec8 protein. Data represents mean ± stdev. * p < 0.05, GFP vs. USP2-45.
concentrations in mice transduced with USP2-45 adenovirus. In fact, the expression of SREBP1c, an insulin-responsive regulator of lipogenesis, is also induced by USP2-45 (Figure 3-5A). AKT phosphorylation is modestly affected by USP2-45 showing increased pAKT-308 phosphorylation while the pAKT-473 levels are similar between both groups (Figure 3-5B). Together, these results demonstrate that USP2-45 augments glucocorticoid signaling in the liver and exacerbates glucose intolerance in insulin resistant state. We next focused on whether USP2 deficiency in high-fat fed mice might have a beneficial affect on glucose tolerance.

3.2.4. Hepatic USP2 knockdown improves glucose homeostasis in obese mice

The expression of USP2 is stimulated by starvation and reaches peak levels at the onset of dark phase (Chapter 2). Both of these conditions are characterized by active glucocorticoid signaling in the liver. As such, it is possible that USP2 plays an important role in modulating local glucocorticoid activation through its regulation of HSD1. We next examined whether RNAi knockdown of USP2 suppresses the gluconeogenic pathway and ameliorates glucose intolerance in HFD-fed mice. We transduced high-fat fed mice with scrambled (Scbl) or USP2 RNAi adenoviruses for 5-7 days. Compared to control, blood glucose levels are significantly lower in mice transduced with siUSP2 adenovirus when measured under ad lib and fasted conditions (Figure 3-6A). In addition,
fasting insulin concentrations are lower in the knockdown group (Figure 3-6B). Liver glycogen content is also reduced in mice transduced with siUSP2 adenovirus, as revealed by quantitative assays and PAS staining (Figure 3-6C&D). We next performed GTT and ITT in transduced mice to assess whole body glucose tolerance and insulin sensitivity. As shown in Figure 3-6E, blood glucose levels following an i.p. glucose bolus are significantly lower in mice transduced with siUSP2 adenovirus. Similarly, USP2 knockdown results in lower blood glucose levels following insulin injection (Figure 3-6F). Extensive serum metabolite analysis also revealed elevated levels of free fatty acids and ketone bodies in fasted siUSP2 mice compared to controls (Table 3-4). This could be compensation for decreased glucose output from the liver. In addition, serum triglyceride content remained similar in each group (Table 3-5). These results demonstrate that depletion of USP2 in the liver improves glucose homeostasis in insulin resistant state.

Gene expression analysis reveals that RNAi knockdown of USP2 results in approximately 65% reduction in USP2 mRNA levels in the liver (Figure 3-7A). The expression of G6Pase and G6PT are decreased in response to USP2 knockdown. Surprisingly, PEPCK expression is only modestly affected, possibly as a result of lower insulin levels in mice transduced with siUSP2 adenovirus. In addition, hepatic expression of TSC22D3 and DUSP1 are decreased following USP2 knockdown. Interestingly, mRNA levels of IGFBP1 and ANGPTL4 remain unaltered by the treatments. Compared to control, HSD1 mRNA (Figure 3-7A)
Figure 3-6. RNAi knockdown of USP2 in the liver ameliorates glucose intolerance in high-fat fed mice. (A) Plasma glucose concentrations in HFD mice transduced with control (filled, n=7) or siUSP2 (open, n=7) adenovirus. (B) Plasma insulin levels in transduced mice following overnight fasting. (C) Liver glycogen content in transduced mice. (D) PAS stain of paraffin-embedded liver sections. (E) Glucose tolerance test and (F) insulin tolerance test in mice transduced with control (filled diamonds) or siUSP2 (open squares) adenovirus. Data represents mean ± s.e.m. * p < 0.05, control vs. siUSP2.
Tables 3-4 – 3-6. Serum metabolites and other physiological characteristics of mice transduced with either Scbl or siUSP2 adenovirus. 

**Table 3.4** Serum free fatty-acids [mM], Ketone Bodies [nM], Serum Cholesterol [mg/dL] 

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<tbody>
<tr>
<td>Scbl</td>
<td>0.453 ± 0.031</td>
<td>0.568 ± 0.045</td>
<td>---</td>
<td>0.702 ± 0.061</td>
<td>74.33 ± 3.9</td>
<td>86.05 ± 4.8</td>
</tr>
<tr>
<td>siUSP2</td>
<td>0.510 ± 0.039</td>
<td>0.857 ± 0.062*</td>
<td>---</td>
<td>1.071 ± 0.16*</td>
<td>73.79 ± 4.5</td>
<td>74.14 ± 3.2*</td>
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**Table 3.5** Free Glycerol [mg/dL], Total [mg/dL], Serum Triglycerides [mg/dL] 

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<tr>
<td>Scbl</td>
<td>---</td>
<td>51.42 ± 4.5</td>
<td>---</td>
<td>103.69 ± 6.7</td>
<td>---</td>
<td>52.27 ± 8.0</td>
</tr>
<tr>
<td>siUSP2</td>
<td>---</td>
<td>47.92 ± 3.0</td>
<td>---</td>
<td>100.22 ± 10.3</td>
<td>---</td>
<td>52.29 ± 10.4</td>
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**Table 3.6** Body Weight (g), LW/BW (%), WATW/BW (%) 

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<tbody>
<tr>
<td>Scbl</td>
<td>41.6 ± 0.62</td>
<td>26.80 ± 0.64</td>
<td>5.5 ± 0.22</td>
<td>5.01 ± 0.11</td>
<td>4.4 ± 0.19</td>
<td>2.71 ± 0.52</td>
</tr>
<tr>
<td>siUSP2</td>
<td>41.9 ± 2.3</td>
<td>26.31 ± 0.44</td>
<td>5.2 ± 0.25</td>
<td>5.00 ± 0.11</td>
<td>4.3 ± 0.60</td>
<td>1.59 ± 0.18*</td>
</tr>
</tbody>
</table>

Data represents mean ± s.e.m. Fed group n = 7; fasting group Scbl, n = 6, siUSP2 n = 7. * p < 0.05 Scbl v. siUSP2.
and protein levels are decreased in mice transduced with siUSP2 adenovirus (Figure 3-7B). Decreased HSD1 protein expression in siUSP2 samples was confirmed using ImageJ software analysis (Figure 3-7C). Again, while HSD1 protein levels were decreased Sec8 and glucocorticoid receptor (GR) protein expression remains similar between both groups. AKT phosphorylation at serine 473 (pAKT-473) while inconsistent in the Scbl group does appear to be lower in the siUSP2 animals - while pAKT-308 levels appear similar between both groups (Figure 3-7B). We conclude from these studies that hepatic USP2 is required for maintaining normal levels of HSD1 expression and glucocorticoid action in the liver. However, many question remain as to the exact mechanism by which UPS2-45 augments glucocorticoid signaling in liver tissue and its role in glucose output. In the next 2 sections we lay out preliminary studies trying to answer those two questions.

3.2.5. USP2-45 is not sufficient to drive glucose secretion in primary hepatocytes

We have now established that when we perturb USP2 levels in vivo, we see a drastic affect on glucose homeostasis. We believe this effect primarily involves changes in glucose flux within the liver, however, we cannot rule out secondary effects by peripheral tissues such as skeletal muscle and white adipose tissue. One way to address this concern is to determine whether USP2-45 can drive glucose secretion in an isolated cell model. We transduced
Figure 3-7. Knock-down of USP2 in liver tissue results in lower expression of HSD1 and several glucocorticoid responsive genes. (A) qPCR analysis of liver gene expression in HFD-fed mice transduced with control (filled) or siUSP2 (open) adenovirus. Data represents mean ± s.e.m. * p < 0.05, control vs. siUSP2. (B) Western blot analysis of total liver lysates from transduced mice. (C) ImageJ quantitation of HSD1 protein levels in panel B normalized to Sec8 protein. Data represents mean ± stdev. * p < 0.05, control vs. siUSP2.
primary hepatocytes with either a low or high dose of GFP control virus or USP2-45. Interestingly, at both concentrations USP2-45 had no effect on glucose secretion compared to control (Figure 3-8A). We then infected primary hepatocytes with siUSP2 and looked at glucose secretion. At this time it must be pointed out that the mRNA level of USP2-45 decreases significantly in isolate primary hepatocytes compared to liver tissue. The cycle number (CT) for USP2-45 in a real-time PCR assay is between 28-30 depending on the hepatocyte prep compared to a cycle number between 19-20 in liver tissue (Chapter 2). We see a similar effect with gluconeogenic genes PEPCK and G6Pase as well as PGC-1α (data not shown). However, since we do not have a good antibody for USP2-45 (Chapter 2) we cannot rule out the possibility that USP2-45 protein levels are significantly higher than mRNA levels would predict. Under this reasoning we proceeded with transducing primary hepatocytes with Ad-siUSP2 and examine glucose secretion. Similar to over-expression of USP2-45, transducing cells with siUSP2 (two doses) had no effect on glucose secretion compared to a scramble (Scbl) control virus (Figure 3-8B). From this line of experiments, we conclude that USP2-45 is not sufficient to increase glucose secretion in vitro. However, it is still unclear whether USP2-45 is required for proper glucose secretion in primary hepatocytes due to its low expression level in culture.

To try to address this shortcoming we looked to increase glucose secretion by co-infecting hepatocytes with PGC-1α – a transcription factor known to regulate the gluconeogenic pathway both in vitro and in vivo [5, 33]. Furthermore, since PGC-1α regulates USP2-45, we postulated that knockdown
of USP2 in the context of PGC-1α over-expression might yield a deficiency in PGC-1α’s function – potentially providing a role for USP2-45. We infected hepatocytes with either GFP or PGC-1α followed by a second infection with GFP or USP2-45. Co-infection of GFP+USP2-45 showed no significant increase in glucose secretion while PGC-1α+GFP increased glucose secretion roughly 3 fold (Figure 3-8C). In addition, co-infection of PGC-1α+USP2-45 yielded no significant increase in glucose output above that which PGC-1α+GFP already induced. Next, to examine whether USP2 is required for PGC-1α induction of glucose secretion, we first infected cells with either GFP or PGC-1α followed by infection with either an Scbl control or an increasing dose of siUSP2. Similar to Figure 3-8C, we saw roughly a 3-fold induction of glucose secretion from cells infected with PGC-1α compare to GFP control (Figure 3-8D). However, we saw no impact on glucose secretion when we co-infected cells with PGC-1α+siUSP2 (at both doses) compared to PGC-1α+Scbl wells. To validate our co-infection model we looked at USP2-45 mRNA levels. We observed roughly a 75% knockdown in USP2-45 mRNA levels in cells co-infected with PGC-1α+siUSP2 compared to PGC-1α+Scbl control (Figure 3-8E). From these experiments we conclude that at least in our primary hepatocyte culture model, USP2-45 alone or in co-infection experiments with PGC-1α is not sufficient to augment glucose output. It also appears as though in respect to PGC-1α’s ability to increase glucose secretion, USP2-45 is dispensable as well. However, since we see such
Figure 3-8. Overexpression or knock-down of USP2-45 does not disrupt glucose secretion in mouse primary hepatocytes. (A) Measurement of glucose secretion in primary hepatocytes transduced with a low or high dose of Ad-GFP or Ad-USP2-45 or (B) with Ad-Scrbl (scramble) or Ad-siUSP2. Glucose secretion in primary hepatocytes co-infected with either (C) PGC-1α and USP2-45 or (D) PGC-1α and siUSP2. Glucose secretion assays were run in duplicate wells for A-D and normalized to protein content within each well using Bradford method. Data represents mean ± stdev. * = p < 0.05. (E) USP2-45 mRNA expression in samples co-infected with Ad-Scrbl and Ad-PGC-1α or Ad-siUSP2 and Ad-PGC-1α. Samples were run in duplicate and represent the mean ± stdev. * = p < 0.05.
a strong phenotype in vivo, it could be that primary hepatocytes do not adequately model the complexity of liver tissue.

3.2.6. HSD1 is de-ubiquitinated by USP2-45 In Vitro

Our model so far for UPS2-45’s action on glucose homeostasis in liver tissue is the localized increase in glucocorticoid signalling via increases in both mRNA and protein levels of HSD1. Since other papers point to USP2’s (both USP2-45 and USP2-69) role in post-translational modification of proteins, we wondered whether HSD1 was also a target of USP2-45. However, we first needed to establish whether HSD1 is ubiquitinated as there are no published reports showing such data. To this end we transfected either Flag-tagged ubiquitin (Flag-Ub) or HA-tagged HSD1 (HA-HSD11β1) separately, or in combination, in Ad293 cells. We then immuno-precipitated with anti-HA beads and blotted against Flag. Surprisingly, when Flag-Ub and HA-HASD11β1 were transfected in the same well we observed both mono- and poly-ubiquitinated HSD1. The presence of poly-ubiquitinated species suggested HSD1 is a target of proteasomal degradation. Indeed, when we treated co-transfected cells with proteasomal inhibitors, MG132 and PS341, we observed an increase in levels of poly-ubiquitinated HSD1 (Figure 3-9A). In contrast, over-expression of wild-type USP2-45 decreased ubiquitination of HSD1 in a dose dependent manner. Inclusion of a catalytically dead USP2-45, USP2-45 H348A, had not effect on ubiquitin levels of HSD1.
**Figure 3-9.** USP2-45 de-ubiquitinates HSD1 *in vitro*. (A) Ad293 cells were co-transfected with either Flag-tagged Ubiquitin (Flag-Ub) or HA-tagged HSD1 or in combination. Samples were then immune-precipitated using anti-HA beads and immune-blotted with anti-Flag. Samples were also treated with proteasomal inhibitors MG132 and PS341 - poly-ubiquitination schematic to right of western blot. (B) Ad293 cells were transfected with different combinations of Flag-Ub, HA-HSD1, USP2-45 (WT) and USP2-45 (H348A) – WT and H348A were titrated into transfected cells. Sample lysates were then immuno-precipitated with anti-HA beads and immuno-blotted using anti-Flag. An anti-tubulin antibody was used to control for protein loading.
We next sought to identify potential lysine residues responsible for ubiquitination of HSD1. The HSD1 protein is intrinsic to the ER membrane, containing a short five-amino acid (M-A-V-M-K) cytosolic domain, followed by a single transmembrane domain, and a catalytic domain located in the ER lumen (Figure 3-10A) [34]. The lysine residue in the cytosolic domain presents an interesting candidate because of its localization as well as it being conserved in other species including; guinea pig, rabbit, rat, monkey and humans. However, loss of this lysine (HSD1 K5R) did not result in loss of ubiquitination in co-transfection experiments with Flag-tagged Ub (Figure 3-10B). We conclude from these experiments that HSD1 is indeed ubiquitinated, at least in vitro, and does not depend on the conserved lysine residue in the cytosolic domain. We also demonstrate that USP2-45 over-expression results in deubiquitination of HSD1. However, what role this has on HSD1 function or stability remains to be determined.

3.2.7 Inhibition of HSD1 by CBX blocks the stimulatory effects of USP2-45 on hepatic gluconeogenesis

To directly assess the significance of HSD1 in mediating the effects of USP2-45 on glucose metabolism, we transduced HFD fed mice with GFP or USP2-45 adenoviruses followed by subcutaneous injection of saline or carbenoxolone (CBX, 40 mg/kg), an inhibitor of HSD1. Chronic CBX treatments have been previously demonstrated to improve glycemic control in rodents and
Figure 3-10. **Cytosolic lysine residue in HSD1 is not required for ubiquitination.** (A) Schematic of HSD1 localization in the ER membrane. HSD1 contains a catalytic domain localized in the ER lumen with a single transmembrane domain and a 5-amino acid cytosolic domain. (B) Ubiquitination assay with cells transfected with either: Flag-Ub, HA-HSD1 WT, HA-HSD1 K5R - or in combination. Samples were immuno-precipitated with anti-HA beads and blotted against Flag. Samples were also blotted against HSD1 to ensure similar protein levels.
humans [35-37]. As expected, adenoviral-mediated expression of USP2-45 significantly elevates circulating glucose and insulin concentrations (Figure 3-11A). While daily injections of CBX for three days have modest effects on blood glucose levels in control mice, these treatments nearly abolished the hyperglycemic effects of USP2-45. Plasma insulin levels are also decreased by CBX in mice with hepatic overexpression of USP2-45. Further, the augmentation of liver glycogen accumulation in response to USP2-45 is significantly blocked by CBX treatments (Figure 3-11B). Analysis of hepatic gene expression indicates that the induction of PEPCK and G6Pase by USP2-45 is significantly diminished by HSD1 inhibition, while surprisingly G6PT1 remains unaltered (Figure 3-11C).

Body weights were similar between all four groups of mice (Table 3-7). However, the liver weights (LW/BW) for USP2-45 (PBS) and USP2-45 (CBX) mice were significantly smaller than that of GFP (PBS) mice. This phenotype was observed in earlier experiment (Section 3.23). Serum metabolite analysis reveals no significant changes in cholesterol (Table 3-8) or free fatty-acid (Table 3-9) levels. However, we do observe a significant decrease in free glycerol levels in USP2-45 (PBS) mice compared to GFP (PBS) mice, which might indicate less lipolysis in white adipose tissue – this would fit as USP2-45 (PBS) mice have significantly higher insulin levels (Table 3-10). White adipose tissue weight (WATW/BW) while elevated in USP2-45 (PBS) mice, does not reach significant levels compared to GFP (PBS) mice.
Figure 3-11. HSD1 inhibition blocks the effects of USP2-45 on glucose metabolism. (A) Plasma glucose and insulin concentrations and (B) liver glycogen content in mice transduced with GFP (open, n=6) or USP2-45 (filled, n=6) followed by treatments with vehicle or CBX for 3 days. Data represents mean ± s.e.m. * p < 0.05 USP2-45 vs. GFP; # p < 0.05 CBX vs. PBS. (C) qPCR analysis of liver gene expression. Data represents mean ± stdev using pooled RNA samples assayed in triplicate.
### Table 3.7

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<th>LW/BW (%)</th>
<th>WATW/BW (%)</th>
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<tr>
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<td>41.6 ± 1.08</td>
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<td>4.57 ± 0.46</td>
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<td>USP2-45</td>
<td>PBS</td>
<td>41.9 ± 0.79</td>
<td>4.36 ± 0.35*</td>
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<td>CBX</td>
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### Table 3.8

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<td>CBX</td>
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### Table 3.9

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<tr>
<td>GFP</td>
<td>CBX</td>
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<td>CBX</td>
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### Table 3.10

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<tr>
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<td>51.2 ± 3.4</td>
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<td>51.1 ± 5.3*#</td>
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**Tables 3-7 – 3-10. Serum metabolites and other physiological characteristics of mice treated with CBX.**

**Table 3-7** Body weights, liver weights (LW/BW) and white adipose tissue weights (WATW/BW) at time of harvest of mice transduced with GFP or USP2-45, followed by a 3d treatment with vehicle or CBX. Liver and white adipose weights are normalized to body weights. **Table 3-8** Serum cholesterol levels at time of harvest. **Table 3-9** Serum free fatty-acid levels at time of harvest. **Table 3-10** Serum triglyceride levels at time of harvest. Data represents mean ± s.e.m. * p < 0.05 USP2-45 vs. GFP; # p < 0.05 CBX vs. PBS.
Together, these results strongly suggest that the activation of glucocorticoid signaling through HSD1 is critical for mediating the metabolic effects of USP2-45 on hepatic gluconeogenesis and glucose production. However, we cannot exclude off target effects of CBX, since it was administered systemically, and more experiments are needed to solidify this inference.

3.3. Conclusions

1.) USP2 is sufficient and necessary for maintaining proper fasting gluconeogenic activity through augmentation of hepatic glucocorticoid signaling.

2.) In high-fat diet fed mice, over-expression of USP2-45 exacerbates glucose intolerance and insulin resistance. In contrast, knockdown of USP2 in liver tissue ameliorates glucose intolerance in similar HFD experiments.

3.) HSD1 induction is required for mediating USP2’s effect on hepatic glucose metabolism.

3.4. Discussion

Hepatic gluconeogenesis is increased in response to starvation and is critical for maintaining a steady supply of glucose for tissues that rely on glucose
for energy production. Glucocorticoids and glucagon are major counter-regulatory hormones that stimulate gluconeogenic gene expression and hepatic glucose production. In mammals, diurnal feeding cycles also provide physiological cues that modulate hepatic glucose and lipid metabolism. In fact, the expression of a large number of genes involved in nutrient and energy metabolism is highly rhythmic in rodent livers [8, 38, 39]. The mechanisms that integrate these hormonal and circadian signals in the regulation of gluconeogenesis are poorly understood. Here, we identified USP2-45 as a deubiquitinase capable of regulating hepatic gluconeogenesis and glucose homeostasis through modulating glucocorticoid signaling in the liver.

*In vivo* gain- and loss-of-function studies demonstrate that USP2 is sufficient and necessary for maintaining normal gluconeogenic activity in the liver. Tail vein injection of recombinant adenovirus expressing USP2-45 leads to elevated blood glucose and insulin concentrations. Direct assessment of hepatic gluconeogenesis using pyruvate as a substrate indicates that USP2-45 significantly increases hepatic glucose production. However, over-expression of USP2-45 in primary hepatocytes did not increase glucose output suggesting other factors are needed for USP2-45 action on glucose homeostasis. Conversely, our primary hepatocyte model might not be adequate to elucidate the effect USP2-45 has on glucose homeostasis in liver tissue. In contrast, depletion of endogenous USP2 by *in vivo* RNAi knockdown impairs gluconeogenic gene expression and results in the development of hypoglycemia during fasting. USP2 deficiency in the liver also perturbs normal diurnal glucose
rhythms under restricted feeding conditions (Figure 3-3). In HFD-fed mice, adenoviral-mediated expression of USP2 exacerbates glucose intolerance and insulin resistance, as illustrated by elevated blood glucose and insulin levels. In this case, hepatic USP2-45 overexpression impairs the ability of transduced mice to clear glucose from circulation during GTT. While it is possible that impaired response to insulin is local in nature, i.e. suppression of gluconeogenesis in the liver, we cannot rule out the possibility that other tissues, such as skeletal muscle and white adipose tissue, may also develop insulin resistance secondary to hepatic insulin resistance. Accordingly, RNAi knockdown of USP-2 ameliorates glucose intolerance in diet-induced obese mice. As such, USP2-45 appears to serve as a “rheostat” in the liver that adjusts hepatic gluconeogenesis and glucose output.

Perhaps the most intriguing aspect of USP2 regulation of hepatic gluconeogenesis is its role in modulating HSD1 expression. Hepatic overexpression of USP2-45 increases HSD1 mRNA and protein levels, leading to the induction of gluconeogenic genes as well as several other glucocorticoid targets, whereas RNAi knockdown of USP2 significantly lowers HSD1 gene expression in the liver. Also it appears HSD1 is a target of ubiquitination and that USP2-45 can modulate this affect. What role that level of regulation plays in HSD1 function or stability still remains to be explored. More importantly, chemical inhibition of HSD1 activity by CBX abolished the ability of USP2-45 to activate gluconeogenic genes and raise blood glucose levels, suggesting that HSD1 induction is required for mediating the effects of USP2 on glucose
3.5. Future Directions

3.5.1. Increased hepatic gluconeogenesis through USP2-45

Over expression of USP2-45 led to an increase in glucose output when mice were challenged with a bolus of pyruvate. In contrast knockdown of USP2 caused a significant decrease in glucose output in a similar pyruvate challenge. However, we also observed significant changes in plasma insulin concentrations, especially in USP2-45 over-expression mice. While a PTT and ITT can give good estimates for hepatic glucose output and insulin sensitivity they are not the gold standard in the metabolic field. A better way to address this question would be to employ a hyperinsulinemic euglycemic clamp study. This will enable us to better measure hepatic glucose output as well as systemic insulin sensitivity while maintaining constant insulin levels.

3.5.2. Glucocorticoid signalling

Glucocorticoid signalling has been shown to affect the gene expression levels of gluconeogenic genes such as PEPCK and G6Pase [40]. In our studies USP2-45 augments gene expression of glucocorticoid responsive genes PEPCK and G6Pase, as well as HSD1, IGFBP1 and TSC22D3. Glucocorticoid receptor levels do not change in whole cell lysates in either USP2-45 over expression or siUSP2 knockdown experiments; however one thing we have yet to look at is GR
localization. Upon ligand binding GR translocates to the nucleus to drive mRNA transcription. One way to look at GR localization would be to isolate nuclei from liver tissue and look at GR content. If USP2-45 is increasing glucocorticoid signalling then we should see an increase in GR nuclear localization.

In addition, previous work has demonstrated insulin resistance and obesity are not associated with systemic increases in glucocorticoid levels but rather local ligand activation via increased activity of HSD1 [41]. In this chapter we postulated that over-expression of USP2-45 augments glucocorticoid signalling by increasing expression of HSD1. This is supported by increases in GC response genes as well as amelioration of USP2-45 hyperglycemia when HSD1 is chemically inhibited (CBX). However, whether HSD1 enzymatic activity, and therefore glucocorticoid activation is actually enhanced in USP2-45 mice remains unknown. HSD1 enzymatic activity could be analysed using isolated liver microsomes from USP2-45 over-expression mice or siUSP2 mice – measuring the rate of conversion of $[^3]$H corticosterone to 11-dehydrocorticosterone. This would be a definitive method showing an increase in HSD1 activity and glucocorticoid signaling.

3.5.3. Ubiquitination of HSD1

For the first time we demonstrate that HSD1 is ubiquitinated, in vitro. However, what role ubiquitination plays in HSD1 function or stability still remains unknown. Initial results suggest HSD1 is poly-ubiquitinated and the USP2-45 is able to reverse this process. The poly-ubiquitination suggests that HSD1 is
targeted for degradation by the proteasomal machinery – proteasomal inhibitors also increased this poly-ubiquitination. We posit that USP2-45 maybe playing a role in HSD1 stability. One way to address this inference is using pulse-chase assays in co-transfection experiments with HSD1, USP2-45 WT and USP2-45 H348A and looking at HSD1 stability. How this fits in with our model still remains to be seen and more experiments are required to address all these questions.

3.6. Materials and Methods

**Cultured primary hepatocytes.** Primary hepatocytes were isolated from C57/Bl6J mice using collagenase type-II (Invitrogen), as previously described [42]. Hepatocytes were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% bovine growth serum (BGS) and antibiotics at 37°C and 5% CO₂. For adenoviral transduction, recombinant adenoviruses were generated using AdEasy adenoviral vector (Stratagene) as previously described [43]. Hepatocytes were transduced for 48 hrs at similar moiety of infection before RNA isolation and gene expression analysis. RNAi sequences are as follows; siUSP2 #1 CGACAGATGTGGAGGAAGT; siUSP #2 AGACCCAGATCCAGAGATA.

**Glucose Secretion Assay.** Primary hepatocytes were cultured as previously described above. Virus was transduced in primary hepatocytes for 48hrs prior to glucose secretion assay. For co-infection experiments primary hepatocytes were transduced with viruses in series. Primary hepatocytes were infected with the
first virus for 1hr, washed with media, and then transduced with second virus for 1hr – cells were again washed and fresh media added. After 48hrs cells were washed with cold 1x PBS three times and placed on gluconeogenic media (phenol red free DMEM, 10mM sodium pyruvate, 2mM D-Lactate). After 3hrs quantification of glucose secretion was analyzed using a Glucose Oxidase/Peroxidase Reagent and o-Dianisidine (Sigma) followed by spectral analysis at 540nm. Cells were again washed twice with 1x PBS and incubated with Flag lysis buffer on ice for 30 minutes. Protein concentrations from cell lysates were measured using Bradford reagent (BioRad).

**Gene expression analysis.** Total liver or hepatocyte RNA was isolated using Trizol (Invitrogen), reversed transcribed using MMLV reverse transcriptase, and analyzed by quantitative PCR using Sybr Green method. The primers used for qPCR analysis are listed: Tsc22d3 (Fwd 5’- cctcaacactgacaagctgaac; Rev 5’- gagcatgtgtcttgctatgttg), Angptl4 (Fwd 5’- gactcagctcaaggctaaaaac; Rev 5’- ttccccctgaagttctgtctac), Dusp1 (Fwd 5’- gatcctgtcctctgtacg; Rev 5’- gtgacctcaagtggttagga), HSD1 (Fwd 5’- ggaggaagtctccagaaggt; Rev 5’- agagacagcaggtctgagtg), TNFα (Fwd 5’- agcccccagtcttgatcttt; Rev 5’- ctccccctgccagaactcagg), CCL2 (Fwd 5’- aggtccctgtcagcttcttg; Rev 5’- tctggacccchctctctcttg), IL6 (Fwd 5’- agttgccttctgggactga; Rev 5’- tccacgatttcgccagaagtt) or described in previous studies [5, 43].
In vivo mouse studies. C57BL/6J mice were kept on a 12:12 light-dark cycle with food and water freely available. For fasting/refeeding studies, mice were provided food ad lib, fasted for 20 hrs, or refed for 18 hrs following fasting. Tissues were harvested at the same time and frozen immediately for gene expression analysis. For in vivo adenoviral transduction, chow or high-fat fed male mice were injected via tail vein purified adenoviruses at approximately 0.15 OD per mouse. Metabolic studies and gene expression analysis were performed 5-7 days following tail vein injection. The expression of GFP and adenoviral gene AdE4 was monitored to ensure similar doses were administered. For carbenoxolone treatments, high-fat diet transduced mice were subcutaneously injected with either saline or 40 mg/kg of body weight or CBX once daily for 3 consecutive days between 11am and 12pm. Bmal1 flox/flox mice were obtained from the Jackson Laboratory. Liver-specific Bmal1 knockout mice were generated by breeding the flox/flox mice with Albumin-Cre transgenic mice. Tissues were harvested every 3 hrs for a period of 24 hrs for gene expression studies. The University Committee on Use and Care of Animals (UCUCA) at the University of Michigan approved all animal procedures performed in this paper.

Pyruvate, insulin, and glucose tolerance tests. For insulin tolerance test, transduced high-fat diet fed (10-12 weeks) mice were placed in clean cages without food for 4-5 hours and injected i.p. with an insulin solution at 0.8 units/kg of body weight. Blood glucose levels were measured before insulin injection and 20, 40, 60, and 120 minutes after injection. For glucose tolerance test,
transduced high-fat diet fed mice were fasted overnight and injected i.p. with glucose (2g/kg, in 0.9% NaCl). Blood glucose levels were measured before injection and at 20, 40, 60 and 120 minutes after glucose injection. For the pyruvate tolerance test, transduced chow fed mice were fasted over-night (~16 hrs) and injected i.p. with 2g/kg of body weight of sodium pyruvate starting at 9am. Blood glucose levels were measured before injection as well as 20, 40, 60, and 120 minutes post injection.

**Liver glycogen measurements.** Liver glycogen levels were measured as previously described [44]. Briefly, a small piece of liver (30-60 mg) was weighed and boiled at 98.5°C in 30% KOH for 30 minutes with occasional vortexing. Then 1M NaSO4 was added to reaction and thoroughly mixed followed by addition of 100% ethanol to precipitate glycogen. Samples were heated briefly and centrifuged at 13,000rpm for 5 minutes at room temperature. Pellets were resuspended in ddH2O and treated with 100% ethanol followed by a 13,000rpm spin for 5 minutes at room temperature (this process was done twice). The pellet was allowed to dry over night before treatment with amyloglucosidase (0.25mg/ml in 0.2M NaOAc, pH4.8). Treatment with amyloglucosidase was allowed to proceed for 3hrs. Quantification of glucose release was analyzed by using a Glucose Oxidase/Peroxidase Reagent and o-Dianisidine (Sigma) followed by spectral analysis at 540nm.
**Histology.** Liver tissue from transduced mice were fixed in 4% paraformaldehyde and processed for paraffin embedding. Paraffin embedded liver sections were stained for glycogen using Schiff's Periodic Acid Staining (PAS) Kit (Polysciences, Inc).
Notes

Portions of this work were included in a paper in preparation entitled: “Ubiquitin-specific protease 2 regulates hepatic gluconeogenesis and diurnal glucose metabolism through 11β-hydroxysteroid dehydrogenase 1,” Matthew M. Molusky, Siming Li, Di Ma, Lei Yu, and Jiandie D. Lin.

M. M. Molusky and J. D. Lin designed the experiments and prepared the manuscripts. S. Li and L. Yu were responsible for the HSD1 ubiquitination assays as well as the protein blotting in Figures 3-5 & 3-7.
3.6. References


Chapter 4

Localization of UPS2-45 to the peroxisome in primary hepatocytes

4.1. Introduction

Peroxisomes, or microbodies, are single membrane-bound organelles found in virtually all higher and lower order eukaryotes. First described in mouse kidney cells, as circular or spherical organelles, peroxisomes are involved in a wide array of biochemical pathways [1]. Central of which are oxidation of fatty acids (FAs) (both $\alpha$- and $\beta$-oxidation) and detoxification of hydrogen peroxide. In fact, compartmentalization of catalase, an enzyme involved in hydrogen peroxide ($\text{H}_2\text{O}_2$) breakdown, along with $\text{H}_2\text{O}_2$ producing enzymes is how this organelle derived its name. Additionally, in humans, peroxisomes serve a role in the
synthesis of cholesterol (although there is some debate about this), bile acids, and plasmogens [2].

Peroxisomes can vary significantly in size and enzymatic content, corresponding to organism, cell type, tissue type and metabolic demand. However, one pathway that seems to be universal from tissue to tissue and from organism to organism is fatty acid oxidation. In lower eukaryotic species like yeast, FA $\beta$-oxidation is solely accomplished by peroxisomes, while in humans and other higher order eukaryotes the role is shared between peroxisomes and mitochondria [2]. In this case peroxisomes play a critical role in lipid metabolism that is not performed by mitochondria. While short-, medium- and long-chain (predominantly) FAs are metabolized in the mitochondria, very long-chain fatty acids (VLCFAs), notably 24:0 and 26:0, can only be handled by peroxisomes. Other types of FAs that rely on peroxisomal $\beta$-oxidation include; branched-chain fatty acids (BCFAs), like pristanic acid, long-chain polyunsaturated fats as well as bile acid intermediates dihydroxycholestanolic acid (DHCA) and trihydroxycholestanolic acid (THCA). While peroxisomes contain a full array of $\beta$-oxidation machinery, most FAs species go through several rounds of $\alpha$- or $\beta$-oxidation, and then, the intermediates are transported to the mitochondria and oxidized to completion [3].

Peroxisomes lack protein-encoding genetic material and therefore must have proteins imported, post-translationally, into pre-existing peroxisomes. Unlike other translocation machinery, (eg the ER and mitochondria),
peroxisomes are able to facilitate transport of folded even oligomeric protein complexes across the membrane and into the peroxisome matrix. The finer points of this translocation still remain a mystery, however, in general it can be thought of as a cycling-receptor system where cargo attaches to a cytosolic receptor, translocates to the peroxisome matrix where the cargo is released and the receptor is cycled back to the cytoplasm. This mechanism is dependent on both ATP and ubiquitin for import and receptor recycling. Two peroxisomal targeting sequences, termed PTS1 and a less frequently used PTS2 have been characterized. The PTS1 is a tri-peptide motif found in the C-terminus with a consensus sequence of (S/A/C)-(K/R/H)-(L/M). While the PTS2 nona-peptide motif is found in the N-terminus of target proteins, with a consensus sequence of (R/K)-(L/I/V)-X₅-(Q/H)-(L/I/V). In each instance target proteins bind to one of two soluble cognate receptors, PEX5 for PTS1 and PEX7 for PTS2, facilitating import across the peroxisome membrane [1]. These soluble receptors are then recycled back to the cytosol to bind with other cargo.

The importance of peroxisomes can be highlighted by an array of genetic diseases that are associated with peroxisomal dysfunction. One of the most notable is Zellweger Syndrome (ZS), a peroxisome biogenesis disorder (PBD), which results in complete absence of peroxisomes in patients. It is characterized by impaired brain development, craniofacial abnormalities and liver dysfunction, resulting in a life expectancy of less than a year. Other notable diseases include X-linked adrenoleukodystrophy (X-ALD), which results in impaired β-oxidation and Refsum disease, which results in impaired α-oxidation and build up of toxic
BCFAs, like phytanic acid. Many of these diseases demonstrate an increase in circulating VLCFAs underlying the importance of peroxisomes in fatty acid oxidation.

4.2. Results

4.2.1. In Silico approach to looking at USP2-45 localization

Localization or compartmentalization is one way in biology to segregate enzymatic function and create specificity. To identify where USP2-45 might localize in the cell we employed an in silico approach. We felt this might elicit a rapid dissemination of information allowing us to narrow down our field of experiments. We used the PSORTII (freely available at ExPASy.com) program developed by Horton & Nakai [4]. This prediction model considers nuclear, cytoplasmic, endoplasmic reticulum (ER) and peroxisomal localization among others for eukaryotic proteins. What this prediction model demonstrated was USP2-45 contains a PTS1 (-SRM) motif in the C-terminus of the protein. Also, since USP2-45 and USP2-69 share a common C-terminal (Chapter 2), USP2-69 also contains this PTS1 motif (-SRM). We next determined whether this was unique to USP2 or whether other USP family members also contained a PTS1 motif. To this end we employed a PTS1 prediction program developed by Eisenhaber et al [5]. This prediction model took into account not only the C-terminus tri-peptide motif, but also nine upstream residues critical for signal recognition by PEX5 (See Introduction).
Figure 4-1  \textit{In silico} peroxisomal targeting sequence 1 (PTS1) screen for all annotated ubiquitin specific proteases (mouse). The PTS1 predictor program developed by Neuberger G \textit{et al.} was used to identify USP family members harboring a C-terminal PTS1 motif. Known PTS1 containing proteins (and peroxisomally located) catalase, ECH1, and EHHADH were used as positive controls.
For this *in silico* screen we used all annotated mouse USP family members as well as catalase, enoyl coenzyme A hydratase 1 (ECH1) and enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (EHHADH) as positive controls for PTS1 motifs. We then plotted the score the query received (positive score correlates with possible peroxisomal targeting and negative score correlates with no peroxisomal targeting) versus an arbitrary number assigned to the USP family member. USP2 received a positive score with an identifiable PTS1 motif suggesting peroxisomal targeting. Interestingly, no other USP family member received a positive score, suggesting this PTS1 motif is unique to USP2. Also, all three positive controls catalase, ECH1, EHHADH were predicted to be peroxisomal giving us some confidence in the prediction model. We conclude from these experiments that USP2 does contain a PTS1 motif and that USP2 is possibly localized to the peroxisome. We next sought to confirm our *in silico* results by physically looking at USP2-45 localization using immunofluorescence.

### 4.2.2 USP2-45 localizes to the peroxisome in primary hepatocytes

While USP2-45 (and USP2-69) contains a consensus PTS1 tripeptide motif (-SRM) this does not necessarily mean USP2-45 is truly peroxisomal. This can be demonstrated by mevalonate kinase (MK) and a bile acid conjugating enzyme, BATT, containing a PTS1 motif, yet are localized to the cytoplasm [2, 6]. To confirm our *in silico* results we looked at USP2-45 localization in primary
Figure 4-2. USP2-45 co-localizes with catalase, a peroxisomal marker, in primary hepatocytes. Immunofluorescent confocal microscopy of primary hepatocytes infected with Ad-USP2-45 and stained for anti-flag, DAPI and either anti-catalase (A), Mitotracker (B) or anti-lamp2 (C). Alexaflour (Invitrogen) secondary antibodies were used in anti-flag and anti-lamp2 images. All three images are merged in far-right column (merged).
hepatocytes using immunofluorescence confocal microscopy. When we used catalase as a marker for peroxisomes we found that it co-localized with flag-tagged USP2-45 (Figure 4.2A, Merged). When we looked at markers for mitochondria (Figure 4.2B) and endosomes (Figure 4.2C) we found no co-localization with USP2-45. Cells were counterstained with DAPI. This suggests that USP2-45 is indeed a bona-fide peroxisomal target. We next chose to interrogate the requirement for USP2-45’s PTS1 motif for peroxisomal targeting.

The PTS1 motif is a cis-acting sequence required for a majority of peroxisomal protein import. Gould et al. demonstrated early on the sufficiency and necessity of this tripeptide motif [7]. To test whether the PTS1 signal is crucial for USP2-45 localization we employed site-directed mutagenesis – utilizing a two-prong approach. We either mutated the serine in the number 1 position (-SRM) to a glutamic acid (S394E), or we inserted a premature stop codon at the serine position (S394Stop), removing the PTS1 entirely. Again, we employed immunofluorescent confocal microscopy to visualize localization of our PTS1 mutant constructs. As anticipated in both cases we observed a cytoplasmic localization for the USP2-45 S394 (Figure 4.3, middle row) and USP2-45 S394Stop (Figure 4.3, lower row) mutants compared to wild-type USP2-45 (Figure 4.3, upper row), which co-localizes with our peroxisomal marker, catalase (merged column). Slides were counter-stained with DAPI. This suggests that USP2-45’s PTS1 motif is required for peroxisomal import, further confirming our previous results.
Figure 4-3. Disruption of PTS1 in USP2-45 causes cytoplasmic distribution in primary hepatocytes. Flag-tagged wild-type (upper), S394E mutant (middle), or S394Stop mutant (lower) USP2-45 constructs were transfected into primary hepatocytes. Cells were fixed and permolized and stained with DAPI (left), anti-catalase (middle left), anti-flag (middle right). All three images are merged on far right.
4.3. Conclusions

1.) USP2-45 contains a peroxisomal targeting sequence (PTS1) and is localized to the peroxisome in primary hepatocytes

2.) The PTS1 motif is required for proper USP2-45 localization.

4.4. Discussion

In mammals, roughly 50 enzymes have been identified as peroxisomal, involving several different metabolic pathways including, polyamine and oxygen metabolism, FA α- and β-oxidation, as well as plasmogen biosynthesis. Our in silico experiments revealed USP2-45 (and USP2-69) to contain a PTS1 motif, and that this motif was unique to USP2 - compared to other family members (Figure 4.1). Here, we have identified USP2-45 as a bona fide peroxisomal protein in primary hepatocytes (Figure 4.2). We further go on to show that the C-terminal PTS1 found in UPS2-45 is critical for its localization to the peroxisome. When the PTS1 motif was disrupted either by point mutation or complete deletion we found USP2-45 localized in the cytoplasm (Figure 4.3). From these experiments it does not seem as though USP2-45 PTS1 mutants affect peroxisome morphology or abundance but detailed experiments analyzing these parameters have not been completed. At this point it is still unclear what role UPS2-45 might be playing in peroxisome function.
One possible function of USP2-45 is changing the ubiquitin state of PEX5, one of the soluble receptors responsible for PTS1 mediated import. As briefly discussed above PEX5 binds to the PTS1 motif of target proteins, where by facilitating cargo transport to the luminal side of the peroxisome – PEX5 is then recycled back to the cytosol. During the cargo unloading and recycling phase PEX5 is mono- or poly-ubiquitinated. In the monoubiquitinated state PEX5 is cycled back to the cytosol (before which point it is deubiquitinated), or in the case of polyubiquination it is thought to be targeted for proteasomal degradation [8]. It must be noted that while evidence points to de-ubiquitination as a key step in recycling the PEX5 receptor back to the cytosol no candidate gene, either in mammals or yeast, have been identified. It is possible that USP2-45 serves in this role regulating the ubiquitination state of PEX5 either saving it from degradation or aiding recycling to the cytosol. At least in the experiments above over-expression of wild-type or mutant USP2-45 in primary hepatocytes does not affect peroxisome import as catalase can still be observed in the peroxisome. In this case more experiments are need to interrogate this line of thinking.

Another possibility is USP2-45 having an affect on peroxisomal fatty acid oxidation. The liver is a key organ for handling fatty acids especially during times of fasting. We also know that over-expression and knock-down experiments in HFD mice does have an affect on serum free fatty acids (FFAs) especially in the fasting state (Chapter 3). However, whether or not this phenotype involves USP2-45 function in peroxisomes is still unclear. One way to answer this
question is to analyze what species of FFAs are affected in these animals, such as VLCFAs, which are known to be elevated in peroxisome disease models.

4.5. Future Directions

While USP2-45 is shown to localize to the peroxisome in primary hepatocytes it is unclear whether USP2-45 also does so in other tissues. It has been demonstrated that the complement of enzymes in peroxisomes can vary from tissue to tissue suggesting some specificity for tissue function. One way to address this would be to use other cell models like C2C12 myotubes or fibroblasts to see if USP2-45 localization still holds true. A similar methodology would be employed to the primary hepatocyte experiments.

Another line of experiments would focus on the possible role of USP2-45 on PEX5 ubiquitination state. As mentioned above ubiquitination of PEX5 is required for proper recycling back to the cytosol. To address this question we would perform similar in vitro ubiquitination assays like the ones carried out for HSD1 in chapter 3. Using co-transfection experiments with wild-type and catalytically dead USP2-45 we could determine whether USP2-45 can deubiquitinate PEX5.

Finally, over-expression of USP2-45 PTS1 mutants might help elucidate the role peroxisomes might be playing in the observed phenotype of USP2-45 in liver tissue. Over expression of Ad-USP2-45 in liver tissue causes an increase in hepatic glucose output as well as decreasing serum free fatty acid levels in HFD
mice. If USP2-45 is affecting these pathways due to its localization to the peroxisome (and therefore peroxisome function in general) PTS1 mutants would be null for this phenotype. However, it is also possible that cytoplasmic localization of USP2-45 (as observed in primary hepatocytes) might cause a plethora of other off target effects confounding the phenotype.

4.6. Materials and Methods

**Immunofluorescence confocal microscopy.** Primary hepatocytes were harvested and cultured as previously described (Chapter 2). However, one notably change was 1° hepatocytes were seed in wells containing collagen coated cover-slips (ethanol and UV sterilized). In Figure 4.2 primary hepatocytes were infected with Ad-USP2-45, fixed and stained with anti-flag and either anti-catalase, mitotracker, or anti-lamp2. For antibody staining, Alexafluor (Invitrogen) secondary antibodies were employed for fluorescent visualization. For Figure 4.3 cells were transfected with wild-type or mutant USP2-45 using polyethyleneimine (PEI, Polysciences, Inc). Staining was similar to as described above.

4.7. References


Chapter 5

Interpretation

5.1 Circadian and metabolic regulation of USP2-45

In the preceding chapters we have started to unravel the mechanisms behind the regulation of USP2. These mechanisms include both circadian and nutritional inputs. The regulation of USP2 is unique for the USP2-45 isoform, as mRNA levels of USP2-69 remain similar under these conditions. So far we have identified several transcription factors important for induction and suppression of USP2-45 expression. In gain-of-function studies we identified both PGC-1α and PGC-1β as potent activator of UPS2-45 transcription. Previous studies have demonstrated that PGC-1α coordinates several aspects of hepatic fasting response, including the activation of gluconeogenesis, heme biosynthesis, and fatty acid β-oxidation [1-3]. However, whether PGC-1α or PGC-1β is required for USP2-45 regulation in a fasting or circadian manner has yet to be thoroughly
explored. This regulation of USP2-45 by the PGC-1 family co-activators seems to be mediated in part by at least two nuclear receptors, HNF-4α and ERRγ. We also identified E4BP4, an insulin regulated transcription factor, as a potent negative regulator of USP2-45 expression. Since we have demonstrated insulin plays a repressive role in USP2-45 regulation this might be mediated thorough E4BP4. We have now identified two potential regulatory arms of USP2-45 potentially integrating both circadian and nutritional signals. However, understanding where all the transcriptions factors fit in still remains unclear.

5.2 Hepatic glucose regulation through ubiquitin-specific protease 2

Hepatic gluconeogenesis is stimulated in response to starvation and is critical for maintaining a steady supply of glucose for tissues that rely on glucose for energy production. Glucocorticoids and glucagon are major counter regulatory hormones that drive gluconeogenic gene expression and hepatic glucose production. In mammals, diurnal feeding cycles also provide physiological cues that modulate hepatic gluconeogenesis. In fact, the expression of PEPCK and G6Pase is highly rhythmic in rodent livers. The mechanisms that integrate these hormonal and circadian signals in the regulation of gluconeogenesis are poorly understood. Here in, we have characterized USP2’s role in liver glucose homeostasis through augmentation of glucocorticoid signaling.

Gain- and loss- of function studies demonstrate that USP2 is required for proper glucose handling in liver tissue. Adenoviral delivery of USP2-45 resulted
in both increased blood glucose and liver glycogen storage. In pyruvate
tolerance tests (PTT) mice transduced with USP2-45 showed increased glucose
output suggesting increased gluconeogenesis. qPCR analysis confirmed over-
expression of USP2-45 drive increases in gluconeogenic genes PEPCK and
G6Pase. Moreover, overexpression of USP-45 in HFD mice results in glucose
intolerance and insulin resistance. In this case, hepatic USP2-45 overexpression
impairs the ability of transduced mice to clear glucose from circulation during
GTT. While it is possible that impaired response to insulin is local in nature, i.e.
suppression of gluconeogenesis in the liver, we cannot rule out the possibility
that other tissues, such as skeletal muscle and white adipose tissue, may also
develop insulin resistance secondary to hepatic insulin resistance. Employing a
hyperinsulinemic euglycemic clamp would help answer this question. In contrast,
siUSP2 transduced mice exhibit suppressed gluconeogenesis and glycogen
storage resulting in fasting hypoglycemia. Interestingly, USP2 deficiency also
results in disruption of normal diurnal glucose rhythms under restricted feeding
conditions. We believe that this increased glucose output is a result of increased
hepatic glucocorticoid signaling. In livers of USP2-45 transduced mice we
observed an increase in glucocorticoid target genes including, IGFBP1,
TSC22D3 and ANGPTL4. Consistent with this we observed increased levels of
HSD1, the enzyme responsible for glucocorticoid ligand activation, at both the
mRNA and protein level. In contrast, USP2 deficiency resulted in decrease
hepatic glucocorticoid signaling and decreased protein and mRNA expression of
HSD1. To demonstrate that HSD1 is downstream of USP2-45 we employed a
chemical inhibitor for HSD1, termed CBX. Over-expression of USP2-45 in mice treated with CBX resulted in decreased blood glucose levels as well as decrease gluconeogenic gene expression. However, whether hepatic over-expression of HSD1 rescues siUSP2 mediate hypoglycemia remains to be explored.

While the exact substrates that mediate the effects of USP2 on HSD1 expression remain unknown at present, it is likely that certain transcription factors and/or cofactors that control HSD1 gene expression may be targeted by USP2-45 in the liver. A potential candidate is C/EBPα, which has been demonstrated to stimulate HSD1 promoter activity [4]. In this case, deubiquitination of C/EBPα and/or other transcriptional regulators by USP2 may result in increased protein stability, leading to the induction of HSD1 expression.

In summary we have identified an ubiquitin-specific protease, USP2-45, that is highly regulated by both nutritional and circadian signals (Figure 5-1). In addition we have characterized USP2's role in hepatic glucose homeostasis through augmentation of the glucocorticoid pathway. However, what role USP2-45 plays in other GR responsive tissues remains to be elucidated. USP2-45 is highly expressed in other tissue and at least in muscle tissue maintains its circadian rhythm. So it is plausible that USP2-45 is playing a similar role in other tissues. What affect this has on glucose homeostasis or other metabolic parameters, has yet to be explored.
Figure 5-1. USP2-45’s role in regulating glucose homeostasis through glucocorticoid activation. The USP2-45 gene product is regulated by both clock and nutritional signals in liver tissue. Our studies have demonstrated a role for USP2-45 in regulating blood glucose levels through control of liver glucose production and output. This increase in glucose production is brought about by USP2-45’s ability to regulate HSD1 mRNA and protein levels, thereby modulating glucocorticoid activation.
5.1 References


