The role of menin in regulation of hepatic glucose production through FoxO1

Leah M. Wuescher

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A Dissertation

entitled

The Role of Menin in Regulation of Hepatic Glucose Production Through FoxO1

by

Leah M. Wuescher

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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December 2012
An Abstract of
The Role of Menin in Regulation of Hepatic Glucose Production through FoxO1

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The menin protein is ubiquitously expressed, but mutations causing loss of menin function lead to a neuro-endocrine specific tumor phenotype called MEN1 Syndrome. Importantly, the MEN1 Syndrome has metabolic manifestations such as glucose intolerance and increased prevalence of type 2 diabetes not associated with the endocrine tumors. Knockout of menin in the liver, a main regulator of glucose homeostasis, yielded no neoplastic phenotype, but no metabolic studies were undertaken. In this collection of studies, our lab shows insulin as a novel regulator of menin expression and localization. Also, insulin facilitates the interaction of menin with FoxO1, the main regulator of gluconeogenesis. To further investigate the purpose of this interaction, mice were created with deletion of one allele of the Men1 gene (HET). On normal chow, mice showed increased markers of FoxO1 activation and when challenged with high fat diet mice showed an exacerbated metabolic phenotype. This evidence supports the hypothesis that menin is a metabolically relevant in the liver with its loss causing an exacerbated metabolic syndrome phenotype.
This work is dedicated to my wonderful husband Christian. You have been my rock throughout this entire process always ready with encouraging words and hugs. Also, to my parents and brother; Charles, Dawn, and Charlie Palladino: Thank you for all of your love and support over the years that got me where I am today.
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Chapter 1

Literature Review

1.1 Liver Physiology

The liver is a vital organ with many functions in maintaining whole body homeostasis. The liver metabolizes administered drugs, creates bile for digestion, and regulates whole body glucose homeostasis with its important role in insulin clearance in feeding, and glucose production in fasting.

1.1.1 The Liver in the Fasted State

The liver is a main relay organ involved in maintaining whole body energy homeostasis (Sommerfeld, Krones-Herzig et al.; Dey and Chandrasekaran 2009). It responds to signals given from the pancreas in the form of secreted hormones to control the peripheral glucose concentration. In times of fasting, circulating levels of glucose are decreased. This decrease triggers pancreatic secretion of glucagon, a peptide hormone, with its receptor being expressed in the liver, kidney, brain, heart, and adipose tissue (Authier and Desbuquois 2008). When glucagon binds its cognate G-protein coupled receptor (GPCR), downstream signaling occurs through cyclic AMP and activation of protein kinase A (PKA). PKA has already been implicated as a negative regulator of
menin protein expression via somatostatin signaling (Mensah-Osman, Zavros et al. 2008). PKA signaling via the glucagon receptor also increases glycogenolysis through phosphorylation of glycogen synthase kinase (GSK) which subsequently activates glycogen phosphorylase leading to increased breakdown of glycogen to glucose (Jiang and Zhang 2003; Ali and Drucker 2009). Glucagon also increases gluconeogenesis after prolonged fasting through regulation of various histone acetyltransferases and histone deacetylase complexes which subsequently affect gene expression (Authier and Desbuquois 2008). Glucagon signaling impedes glucose breakdown (glycolysis) through the inhibition of pyruvate dehydrogenase, thereby stopping the conversion of pyruvate to acetyl CoA allowing it to be used as a substrate for gluconeogenesis (Authier and Desbuquois 2008). Gluconeogenesis is regulated by several signaling molecules, with the most studied being forkhead box transcription factor O1 (FoxO1). FoxO1 has been vastly studied since its discovery in 1995 (Davis, Bennicelli et al. 1995; Fredericks, Galili et al. 1995), with an established role in regulation of gluconeogenesis (Nakae, Kitamura et al. 2001; Puigserver, Rhee et al. 2003). In the fasted state, FoxO1 is predominantly nuclear leading to increased transcription of target genes. FoxO1 directly regulates transcription of Glucose-6-Phosphatase (G6Pase), the rate limiting enzyme and final step of hepatic glucose production. It has also been implicated in increased transcription of phosphoenolpyruvate carboxykinase (PEPCK), another rate limiting step in the pathway of glucose production. Regulation of both of these enzymes is necessary for maintaining normal glucose balance in times of prolonged fasting. Another important consequence of glucagon signaling is the induction of lipolysis in white adipose tissue (Ali and Drucker 2009; Heppner, Habegger et al. 2010). When lipolysis occurs, free fatty acids travel
through the blood to organs such as the muscle and liver to be used for energy through beta oxidation and the TCA cycle leading to ATP production (Sommerfeld, Krones-Herzig et al.).

1.1.2 The Liver in the Fed State

During feeding, the pancreas secretes insulin, a peptide hormone, which influences the adipose tissue, muscle and liver to undergo glucose uptake for storage as glycogen or fatty acids for later use (Gross, van den Heuvel et al. 2008). Circulating insulin during feeding is cleared by the liver primarily, and to a lesser extent, the kidney (Duckworth, Bennett et al. 1998). When insulin binds its receptor in the liver, the receptor undergoes endocytosis and is dissociated from insulin, which then undergoes degradation (Duckworth, Bennett et al. 1998; Poy, Yang et al. 2002). Also, when insulin binds its receptor, the receptor becomes autophosphorylated and can subsequently induce a signaling cascade via phosphotidylinositol 3’ kinase (PI3K) with activation of Akt/PKB (Sommerfeld, Krones-Herzig et al.; Kido, Nakae et al. 2001). Phosphorylation of Akt downstream of the insulin receptor causes multiple effects such as phosphorylation of glycogen synthase kinase 3 (GSK3) causing its inactivation leading to the activation of pathways involving glycogen synthesis, protein synthesis, and fatty acid synthesis (Rayasam, Tulasi et al. 2009). Akt also increases phosphorylation of forkhead box family of transcription factors, notably FoxO1 (Kido, Nakae et al. 2001; Ramnanan, Edgerton et al. 2010). Phosphorylation of FoxO1 leads to its cytoplasmic translocation and inactivation, however, p300 mediated acetylation has also been recently implicated in FoxO1 subcellular localization, (Perrot and Rechler 2005; van der Heide and Smidt 2005; Qiang, Banks et al. 2010). Increased acetylation is associated with cytoplasmic
localization in combination with phosphorylation, while decreased acetylation allows for nuclear localization and increases in FoxO1 transcriptional targets (Qiang, Banks et al. 2010; Banks, Kim-Muller et al. 2011). Inactivation of FoxO1 via translocation from the nucleus to the cytoplasm leads to the suppression of gluconeogenic genes and the expression of genes involved in glycogen synthesis and storage (Kido, Nakae et al. 2001; Zhang, Patil et al. 2006). Another pathway activated by insulin secretion in the liver is glycolysis. Glycolysis is the breakdown of glucose by the liver into pyruvate which also can be converted to lactate or acetyl CoA which subsequently enters the TCA cycle to produce large amounts of ATP (Zhang, Patil et al. 2006). This process is regulated, in part, by glucokinase (GK), the enzyme responsible for the conversion of glucose to glucose 6 phosphate. Glucose 6 phosphate can then undergo further processing through the pathway until it reaches the result of pyruvate. FoxO1 has also been shown to exert effects on the glucokinase promoter in concert with hepatocyte nuclear factor 4 alpha (HNF4α) to regulate its expression in the fasted and fed states (Hirota, Sakamaki et al. 2008).

1.1.3 FoxO1 as a Regulator of Fasting and Feeding

FoxO1 is a member of the forkhead box family of transcription factors characterized by conserved DNA binding domain and an N-terminal region containing an Akt phosphorylation site (Cheng and White 2010). FoxO1 has an established role in maintaining the balance between the gluconeogenesis and glycolysis pathways in times of fasting and feeding (Liu, Dentin et al. 2008; Haeusler, Kaestner et al. 2010). FoxO1 localization is controlled by PKB/Akt signaling downstream of the insulin receptor.
(Gross, Wan et al. 2009). In times of feeding, insulin signaling is high, along with activation of Akt, which causes phosphorylation of FoxO1 and its chaperone, 14-3-3 (Nakae, Oki et al. 2008; Gross, Wan et al. 2009). This phosphorylation causes FoxO1 to be sequestered to the cytoplasm, down-regulating its transcriptional effects (Zhang, Patil et al. 2006; Gross, van den Heuvel et al. 2008; Nakae, Oki et al. 2008; Gross, Wan et al. 2009; Cheng and White 2010). In times of reduced Akt signaling (i.e. fasting) FoxO1 is nuclear and exerting effects on various target genes such as the transcriptional regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), and enzymes that catalyze rate limiting steps in gluconeogenesis glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Puigserver, Rhee et al. 2003; Samuel, Choi et al. 2006; Cheng and White 2010; Oiso, Furukawa et al. 2010; Park, Kim et al. 2010; Ramnanan, Edgerton et al. 2010). FoxO1 binds insulin response elements (IREs) on various genes exerting opposing effects from insulin signaling (Cheng and White 2010). An interesting finding is that FoxO1 binds the insulin receptor promoter and increases transcription and expression of the insulin receptor, thought to prepare cells for the next cycle of insulin secretion/feeding (Puig and Tjian 2005). The effects of FoxO1 that have been studied have been observed in mice exhibiting insulin resistance or have some exogenous modification (i.e. siRNA, overexpression) (Samuel, Choi et al. 2006; Zhang, Patil et al. 2006; Gross, Wan et al. 2009; Cheng and White 2010). *The physiological contribution of FoxO1 in regulation of hepatic metabolism is still not entirely clear* (Zhang, Patil et al. 2006; Gross, van den Heuvel et al. 2008; Gross, Wan et al. 2009; Cheng and White 2010).

1.2 Metabolic Syndrome and T2DM
Metabolic syndrome (MetS) has been defined as involving 5 characteristics: dyslipidemia, abdominal obesity, hypertension, hyperglycemia, and insulin resistance (Grundy, Brewer et al. 2004). MetS is occurring more and more frequently recent years attributing to overnutrition and sedentary lifestyle (Ford, Kohl et al. 2005; Ford, Li et al. 2010). This is a serious issue due to the significant increase in risk of development of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (Grundy, Brewer et al. 2004). T2DM is characterized by impaired glucose tolerance and hyperinsulinemia. This disease is multi-faceted involving many organ systems and overall changes in glucose homeostasis. There is an increased importance for prevention of disease onset and finding possible treatments since T2DM accounts for 90% of all diabetes worldwide and the incidence will double by 2025 (Doria, Patti et al. 2008).

1.2.1 Increased FoxO1 Activity promotes MetS and T2DM Phenotype

The inability of organs to respond to insulin signaling is crucial for development of T2DM. The lack of insulin signaling in the liver leads to lack of insulin clearance, thereby increasing peripheral insulin concentrations (Poy, Yang et al. 2002). Higher peripheral insulin concentrations cause other insulin responsive organs, such as the muscle and adipose, to also become resistant. The muscle is responsible for the approximately 80% of glucose uptake induced by insulin signaling (de Lange, Moreno et al. 2007). This uptake occurs through insulin induction of GLUT4 translocation from the cytoplasm stored in vesicles to the cell membrane. Loss of glucose uptake leads to increased serum glucose, owing to hyperglycemia. Insulin resistance in adipose is characterized by decreased uptake of triglycerides from the serum, partially attributing to
the increased dyslipidemia (Gallagher, LeRoith et al. 2010). Also, insulin resistance in
the adipose causes more release of free fatty acids (FFA) which can be used by other
organs such as the liver for increasing glucose production (Gallagher, LeRoith et al.
2010). In insulin resistance, many major signaling cascades are not functioning properly,
leading to the defects seen in T2DM. FoxO1 has been mostly studied under the influence
of insulin resistant states. Loss of function of hepatic FoxO1 has shown maintenance of
normoglycemia in db/db mice that were insulin resistant (Qu, Altomonte et al. 2006).
This lead to the discovery of the FoxO1 targets PEPCK and G6Pase, both rate-limiting in
hepatic glucose production. Similarly, gain-of-function studies involving
phosphorylation defective mutants of FoxO1 implicate it in exacerbation of the metabolic
syndrome phenotype (Gross, Wan et al. 2009; Cheng and White 2010). FoxO1 has also
been shown to be regulated via acetylation along with phosphorylation (Perrot and
Rechler 2005; van der Heide and Smidt 2005), with acetylation actually seeming to
regulate FoxO1 subcellular localization more than phosphorylation (Qiang, Banks et al.
2010). As previously stated, insulin resistant states are usually accompanied by
hyperglycemia. Acetylation of FoxO1 is regulated both by CBP/p300 and the
deadacetylase Sirtuin1 (SIRT1) leading to deacetylation of FoxO1 and nuclear localization
(Hirota, Sakamaki et al. 2008; Houtkooper, Pirinen et al. 2012). Most recently published
has been the differences in expression of FoxO1 targets depending on if it is a
phosphorylation defective mutant versus an acetylation defective mutant (Banks, Kim-
Muller et al. 2011). This increases the importance of finding FoxO1 binding partners and
seeing how they can affect FoxO1 post translational modifications and function.
1.2.2 Increased FoxO1 Activity Promotes Lipogenesis and Hypertriglyceridemia

Hypertriglyceridemia and nonalcoholic fatty liver disease (NAFLD) are common pathologic manifestations of obesity and insulin resistance as seen in type 2 diabetes (Chavez-Tapia, Uribe et al. 2009; Subramanian and Chait 2012). However, the pathways controlling these manifestations are still not completely known. Insulin resistance in the liver is characterized by inability for the liver to suppress gluconeogenesis and promote glycogen synthesis. Conversely, in insulin resistance hepatic lipogenesis increases, which is typically a sign of insulin sensitivity (Brown and Goldstein 2008). This is the conflict that lead to the hypothesis of “selective insulin resistance” (Brown and Goldstein 2008). Researchers have been trying to identify the regulatory element involved in this phenomenon and recent studies have implicated FoxO1 in regulation of this phenotype.

In humans with NAFLD, FoxO1 was reported to be more active with increased fat deposition in the liver (Valenti, Rametta et al. 2008). In mice with constitutively active FoxO1 it was shown that there was increased expression of lipogenic genes such as SREBP1c, ACC, and FAS (Matsumoto, Han et al. 2006; Zhang, Patil et al. 2006). FoxO1 has also been shown to regulate hepatic lipid metabolism and contribute to hypertriglyceridemia through its regulation of rate limiting enzymes involved in triglyceride synthesis (Sparks and Dong 2009). FoxO1 directly binds the promoter of microsomal transfer protein (MTP) which is responsible for transporting lipids to apoB, a rate limiting step in triglyceride synthesis (Sparks and Sparks 2008). FoxO1 also increases apolipoprotein CIII which acts as an inhibitor of lipoprotein lipase (LpL), protecting newly formed triglyceride from hydrolysis (Kim, Zhang et al. 2011). Increased activity of FoxO1 contributes both to the defects in regulation of hepatic
glucose production and regulation of de novo lipogenesis, this research points to the importance of FoxO1 and its potential for therapeutic intervention in metabolic syndrome and type 2 diabetes.

1.3 Multiple Endocrine Neoplasia Type 1

Multiple Endocrine Neoplasia Type 1 (MEN1) Syndrome is an autosomal dominant disorder involving the development of neuro-endocrine tumors by the 5th decade with near 100% penetrance (Chandrasekharappa, Guru et al. 1997; Shen and Libutti 2010). The protein product of the MEN1 gene, menin, has been characterized as a tumor suppressor because the syndrome follows Knudson’s classic 2-hit hypothesis consisting of one germline mutation followed by tumor development after a somatic mutation. Tumors mainly occur in the anterior pituitary, endocrine pancreas and parathyroid which exert a wide variety of hormonal symptoms (Lemos and Thakker 2008; Tsukada, Nagamura et al. 2008). A striking feature of the MEN1 syndrome is the increased incidence of glucose intolerance and type 2 diabetes (Wagner, Martin-Campos et al. 2005; McCallum, Parameswaran et al. 2006; van Wijk, Dreijerink et al. 2011). Studies of the MEN1 gene and menin protein have not helped to elucidate a genotype-phenotype correlation for MEN1 mutations, the selectivity of the tumor development, or apparent metabolic symptoms (Lemos and Thakker 2008; Tsukada, Nagamura et al. 2008).

1.3.1 The Menin Gene and Protein

The MEN1 gene is located on chromosome 11q13 in humans consisting of 10 exons with the coding region found on exons 2-10 (Chandrasekharappa, Guru et al. 1997; Guru,
Crabtree et al. 1999; Balogh, Patócs et al. 2010). Homologues have been found in mouse, rat, zebrafish, and Drosophila melanogaster (Shen and Libutti 2010). Menin total body heterozygotes developed a tumor profile similar to MEN1 after 9 months of age (Bertolino, Tong et al. 2003). Since homozygous deletion of whole body menin is embryonic lethal, menin conditional knockdowns and knockouts using the Cre/loxP system of deletion have been studied in mice (Bertolino, Radovanovic et al. 2003; Scacheri, Crabtree et al. 2004; Chen, Yan et al. 2006; Shen, He et al. 2009; Cheng, Yang et al. 2011; Wuescher, Angevine et al. 2011; Wuescher, Angevine et al. 2012).

Knockouts in the endocrine pancreas did indeed cause tumor formation, but at 10-15 months of age (Bertolino, Tong et al. 2003; Crabtree, Scacheri et al. 2003; Shen, He et al. 2009). Since menin is expressed ubiquitously and at high levels in the liver, a targeted homozygous deletion of Men1 was developed specifically in the liver (Scacheri, Crabtree et al. 2004). Although there was no hepatoma formation, and no differences in life-span, no metabolic parameters were measured, still leaving the question of increased incidence of type 2 diabetes in these patients. This supports our hypothesis that menin is playing a metabolic regulatory role in the liver. The mRNA product of the MEN1 gene is 2.8 kilobases in length, with 6 alternative transcripts only differing in the 5’-UTR (Bassett, Rashbass et al. 1999; Fromaget, Vercherat et al. 2003). Little is known about the transcriptional regulation of MEN1, but the protein product menin can influence the transcription of the MEN1 gene as a compensatory effect when function of one allele is lost, and we have previously shown that insulin regulates menin at the transcriptional level; most recently, chronically high glucose was shown to decrease menin in the pancreatic β cell (Fromaget, Vercherat et al. 2003; Zablewska, Bylund et al. 2003;
Wuescher, Angevine et al. 2011; Zhang, Li et al. 2012). Also, transcriptional regulation depends on cell type (Fromaget, Vercherat et al. 2003; Zablewska, Bylund et al. 2003). The protein product of MEN1 is a 67 kilodalton protein termed menin which was first cloned in 1997 and shares 97% identity with both the mouse and rat menin (Chandrasekharappa, Guru et al. 1997; Shen and Libutti 2010). Total knockout of menin is embryonic lethal between days 11-13 while the hemizygous deletion yields a phenotype similar to that of the human MEN1 syndrome with development of full neuro-endocrine tumors at 16 months of age with all tumors showing loss of murine Men1 (Crabtree, Scacheri et al. 2001; Crabtree, Scacheri et al. 2003). The menin protein is unique because it shares no homology with any other known protein (Balogh, Rácz et al. 2006; Balogh, Patócs et al. 2010; Huang, Gurung et al. 2012). The protein consists of 5 GTPase sites which only show GTP binding activity in the presence of nm23, a suppressor of tumor metastasis (Yaguchi, Ohkura et al. 2002). Menin also contains 3 nuclear localization signals (NLSs) and multiple phosphorylation sites in the C-terminus of the protein (MacConaill, Hughes et al. 2006; Shen and Libutti 2010; Francis, Lin et al. 2011). Mutations of all 3 of the NLSs do not prevent menin localization to the nucleus, but affect its ability to elicit transcriptional effects on target genes in vitro (La, Desmond et al. 2006). A study of pancreatic tumors in humans revealed that most of the MEN1 mutations were truncation mutations causing loss of the C-terminal region that includes the NLSs and caused cytoplasmic localization (Corbo, Dalai et al. 2010). Mutations of both of the phosphorylation sites on menin did not affect its ability to associate with the mixed lineage leukemia (MLL) histone methyltransferase complex, but no other possible consequences were studied (MacConaill, Hughes et al. 2006). More recently, consistent
with menin’s tumor suppressor function, it has been shown to be phosphorylated in response to DNA damage (Francis, Lin et al. 2011). This phosphorylation was shown to be required for menin dependent transcription of DNA damage response genes. To further elucidate the function of the menin protein, the crystal structure was recently discovered (Huang, Gurung et al. 2012). The structure revealed a binding pocket containing 3 tetratricopeptide motifs which are responsible for protein-protein interactions. It was shown that menin, which binds both JunD and MLL in the same pocket, can regulate them differently. Menin suppressed JunD function while enhancing MLL activity. Even with the discoveries of these functional motifs, there is still no definitive physiological function for menin.

1.3.2 Menin Interacting Proteins (MIPs)

Although the physiological function of menin has not yet been elucidated, there have been many proteins identified that do interact with menin indicating functions related to cell cycle regulation, apoptosis, transcriptional regulation, and most recently, metabolism. A major protein involved in regulation of growth and metabolism is PKB/Akt found solely in the cytoplasmic compartment (Wang, Ozawa et al. 2010). Recently 2 papers have been published showing menin’s regulation of the Akt pathway looking solely at the proliferative consequences and showing menin as a negative regulator of Akt signaling (Gao, Feng et al. 2010; Wang, Ozawa et al. 2010). Both the MAPK and Akt pathways are activated down-stream of insulin receptor activation indicating that insulin receptor signaling could require menin for a feedback mechanism. The most studied MIP is JunD and the mechanism that menin can suppress its activation (Manickam, Vogel et al. 2000;
Kim, Lee et al. 2003; Huang, Gurung et al. 2012). Importantly, beside the fact that menin does bind JunD, menin also can recruit a histone deacetylase complex and act a co-repressor through association with mSin3A (Kim, Lee et al. 2003). Conversely, menin has also been shown to associate with mixed lineage leukemia (MLL) in the trithorax family of proteins with histone methyltransferase (HMT) activity (MacConaill, Hughes et al. 2006; Caslini, Yang et al. 2007; Huang, Gurung et al. 2012). With both acetylation and methylation being key epigenetic regulators of transcription, menin plays a major role in the transcription of various genes. Menin also directly interacts with several proteins such as Smad3 (TGFβ pathway) and NFκB (Zindy, L'Helgoualc'h et al. 2006) affecting target gene transcription (Heppner, Bilimoria et al. 2001; Kaji, Canaff et al. 2001; Theillaumas, Blanc et al. 2008). Menin has also been shown to regulate transcription factors downstream of MAPK signaling (Gallo, Cuozzo et al. 2002). While menin has been classified as a nuclear protein, it does interact with cytoplasmic proteins involved in cell cycle regulation such as vimentin and glial fibrillary acidic protein (GFAP) (Balogh, Rácz et al. 2006; Balogh, Patócs et al. 2010).

1.3.3 Hepatic Menin

Menin plays a role in the liver starting during development. Men1 null pups are embryonic lethal at day E11-13.5. During this timeframe, the liver undergoes its highest phase of growth as it develops into the main hematopoietic organ of the fetus (Zorn 2008). Although Men1 null pups have cranio-facial defects, they also show massive hemorrhage in the abdominal cavity with abnormal liver development (Bertolino, Radovanovic et al. 2003; Lemos, Harding et al. 2009). Tissue specific knockout of menin in the liver was analyzed in the context of tumor suppression, showing loss of
menin was well tolerated (Scacheri, Crabtree et al. 2004). However, no metabolic parameters were measured. Recently, there has been increasing interest in hepatic menin due to its involvement in regulation of fibrogenesis in hepatocellular carcinoma via regulation of the collagen type 1 promoter (Zindy, L'Helgoualc'h et al. 2006). Menin has now been implicated in regulation of PPARα mediated control of beta oxidation to prevent hepatic lipid accumulation (Cheng, Yang et al. 2011). Importantly, our contribution to the growing field of research is the involvement of menin with FoxO1, a main regulator of the switch between fasting and feeding metabolism (Wuescher, Angevine et al. 2011). Additionally, after high fat challenge with tissue specific knockdown of menin, we saw an exacerbation of metabolic syndrome phenotype indicating not just a role for menin in regulation of beta oxidation, but whole body glucose metabolism (Wuescher, Angevine et al. 2012).

1.3.4 Summary and Hypothesis

As previously discussed, the menin protein is ubiquitously expressed but poorly understood. An abundance of research has been dedicated to finding the key to the neuroendocrine tissue specificity of the MEN1 Syndrome phenotype, but little has been designated to the metabolic aspects of the disease. Although a liver specific knockout has been achieved, again, there were no studies on any sort of metabolic phenotype. As FoxO1 is a main regulator of gluconeogenesis in the liver and aberrant FoxO1 activation is associated with metabolic syndrome phenotype, it was used as an initial target of the menin protein. Based on evidence that the MEN1 Syndrome is accompanied by a metabolic phenotype, and that the liver is a main regulator of glucose homeostasis via
FoxO1, we hypothesize: *Hepatic menin is a metabolically relevant protein due to mediating effects on gluconeogenesis through regulation of FoxO1.*
Chapter 2

Insulin regulates Menin expression, cytoplasmic localization and interaction with FOXO1.

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Abstract:

Menin is the ubiquitously expressed nuclear protein product of the MEN1 gene, which interacts with PKB/AKT in the cytoplasm to inhibit its activity. This study describes a novel insulin-dependent mechanism of menin regulation and interaction with other metabolic proteins. We show that insulin down-regulated menin in a time-dependent manner via the human insulin receptor. Inhibition analysis indicated a critical role for the protein kinase AKT in regulation of menin expression and localization. Insulin mediated decrease in menin expression was abrogated by the PI3K/AKT inhibitor, LY294002 at early time points, from 2 hours until 7 hours. Furthermore, exposure to insulin resulted in the cytoplasmic localization of menin and increased interaction with FOXO1. Fasting followed by refeeding modulates serum insulin levels, which corresponded to an increase in menin interaction with FOXO1 in the liver. Liver specific hemizygous deletion of menin resulted in increased expression of FOXO1 target genes, namely IGFBP1, PGC1α, insulin receptor, AKT and G6Pase. This study provides evidence that menin expression and localization is regulated by insulin signaling, and that this regulation triggers an
increase in its interaction with FOXO1 via AKT with metabolic consequences.

**Introduction:**

Menin is the protein product of the multiple endocrine neoplasia type 1 (MEN1) gene with its loss causing a syndrome characterized by development of neuroendocrine tumors. Menin has been shown to interact with key transcriptional elements such as NF-kB, Smad 3 and JunD, to regulate expression of their target genes (1) (11) (12). Menin is ubiquitously expressed in all tissues, both endocrine and neuro-endocrine,(23) and has been described as a tumor suppressor gene in all organs except the liver (21). Previous reports have described a high prevalence of impaired fasting glucose and diabetes mellitus in families with MEN1 inheritance (15). Karnik et al implicated menin in the development of gestation-induced diabetes and associated the loss of menin in beta cells of pancreatic islets to increased proliferation (13).

Menin regulates transcription factors downstream of mitogen activated protein kinase (MAPK) signaling (9) and interacts with cytoplasmic proteins involved in cell cycle regulation (6). A major protein involved in regulation of growth and metabolism is protein kinase B (PKB/AKT). This important serine kinase, found in the cytoplasmic compartment, is inactivated by menin via direct interaction (24). Both the MAPK and AKT pathways are activated down-stream of insulin receptor signaling indicating menin could be involved in the regulation of this signaling cascade.

Although the physiological role of menin has not yet been elucidated, there have been many proteins identified that interact with menin alluding to functions related to cell cycle regulation, apoptosis and transcriptional regulation.
The transcription factor FOXO1 plays a central role in the hepatic regulation of key metabolic genes involved in glucose and fatty acid metabolism, as well as cellular growth and differentiation (10, 22). The transcriptional activity of FOXO1 is regulated by insulin via AKT. In the presence of insulin, FOXO1 is inactivated by AKT through phosphorylation resulting in the translocation of FOXO1 into cytoplasm (14). Deregulated expression and activity of FOXO1 protein results in secondary insulin resistance (8). Fasting and re-feeding acutely modulate insulin levels to activate genes involved in glucose production and fatty acid metabolism. In times of feeding and high AKT activity, FOXO1 is exported from the nucleus into the cytoplasm (20). In the liver, FOXO 1 mediates the switch from glycolysis and carbohydrate metabolism to gluconeogenesis with Glucose-6-Phosphatase (G6Pase) enzyme playing a rate-limiting role (4).

In this study we demonstrate that insulin is a direct regulator of menin expression via the AKT pathway and modulates interaction of menin with FOXO1 in primary hepatocytes and HepG2 cells. We identified that fasting and refeeding regulate the expression of menin in the liver and is also associated with increased interaction of menin with FOXO1 in vivo. We further implicate hepatic menin in glucose metabolism since the hemizygous deletion in the liver induces overproduction of G6Pase, IGFBP1, PGC1α, which are FOXO1 target genes, involved in hepatic glucose production. Furthermore, liver specific menin hemizygous mice display reduced glucose levels during glucose tolerance tests while insulin tolerance testing showed no change. These results provide evidence that menin has a metabolic role in the liver whose interaction with FOXO1 increases during refeeding and is regulated by insulin.
Experimental procedures

**Animal Maintenance:** Animals were kept in a 12-hour dark/light cycle and fed standard chow *ad libitum*. All procedures were approved by Institutional Animal Care and Utilization Committee at the University of Toledo. Liver specific menin hemizygous mice on a mix of FVB/129S mice expressing loxP sequences on exons 3-10 of the Men1 gene and C57/Blk6 mice expressing *Albumin-Cre* were crossed together and genotyping performed with tail lysates. Wild-type (WT) and Flox (loxP) sequences with Cre expression were considered hemizygous for menin in the liver (HETs). Genotyping: Primers A+G give the WT allele, primers F+G give the floxed allele. The primer sequences are as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Men1</td>
<td>5’ – TCC AGT CCC TCT TCA GCT TC – 3’</td>
</tr>
<tr>
<td>F-Men1</td>
<td>5’ – GCC ATT TCA TTA CCT CTG CCG – 3’</td>
</tr>
<tr>
<td>G-Men1</td>
<td>5’ – TAC CAC TGC AAA GGC CAC GC – 3’</td>
</tr>
<tr>
<td>Cre Forward</td>
<td>5’ – TGA GGT TCG CAA GAA CCT GAT GGA – 3’</td>
</tr>
<tr>
<td>Cre Reverse</td>
<td>5’ – GCC GCA TAA CCA GTG AAA CAG CAT – 3’</td>
</tr>
</tbody>
</table>

Tail snips were collected from mice at 21 days after birth digested in a mixture of direct PCR, tail lysis buffer (Thermo Fisher Scientific, Pittsburgh PA) plus Proteinase K (Roche, Indianapolis, IN) overnight, rotating at 55°C. Lysates were collected incubated at 85°C for 45 minutes. The lysates then underwent PCR with the following conditions:
The PCR running conditions for the *Men-1* gene: An initial step of 95˚C for 15 min followed by 35 cycles of denaturing at 94˚C for 30s, annealing at 62˚C for 30s, and extension at 72˚C for 1m30s with a final step of 1 cycle of 72˚C for 10min and hold at 4˚C.

The PCR running conditions of the *Cre* gene: An initial step 95˚C for 15 min followed by 35 cycles of denaturing at 94˚C for 30s, annealing at 66.6˚C for 30s, and extension at 72˚C for 1m30s with a final step of 1 cycle of 72˚C for 10min and hold at 4˚C.

PCR products were run on a 1% agarose gel in TAE for 30min at 120V.

Mice were euthanized at 25 weeks of age.

**Cell culture.** Primary hepatocytes were isolated from livers of WT mice according to the protocol (19) and HepG2 cells were maintained at 37°C and 5% CO2 in DMEM medium containing 10% FBS and penicillin and streptomycin. All experiments were performed on 80% confluent cells.

**Transfections.** Stable transfection of NIH 3T3 cells with the human insulin receptor have previously been described (7).

**Western blot.** The concentration of proteins in tissue and serum lysates was quantitated by BCA protein assay (Pierce, Rockford IL), prior to analysis by 10% or 4-12% gradient SDS-PAGE (Invitrogen, Carlsbad CA), respectively, and immunoprobing with specific antibodies. These include polyclonal antibodies against Menin (AbCam, Cambridge MA and Santa Cruz Biotechnology Inc, Santa Cruz CA), FOXO1, (Santa Cruz Biotechnology Inc, Santa Cruz CA), pERK and ERK (Cell Signaling, Beverly, MA), in addition to
monoclonal antibodies against GAPDH and Lamin B (Sigma-Aldrich, St. Louis MO). Proteins were detected by Odyssey INFRA-Red imaging system using corresponding secondary antibodies conjugated to near infrared dyes.

**Cellular Fractionation.** Cells were fractionated using the NE-PER nuclear protein extraction kit (Thermo Scientific, Rockford, IL) according to manufacturer’s protocol. Briefly, cells were harvested via trypsin-EDTA (Invitrogen, Carlsbad CA) and pelleted at 500xg for 5 minutes (all centrifuge steps at 4˚C). Supernatant was removed, 200ml of ice cold CERI (cytoplasmic extraction reagent 1) added to pellet (supplemented with protease and phosphatase inhibitors) and vortexed vigorously for 15s. Mixture was incubated for 10min prior to adding 11μl of CERII (cytoplasmic extraction reagent 2), vortexed for 5s. Mixture was Incubated on ice for 1min, vortexed again and centrifuged for 5min at max speed. Supernatant (cytoplasmic fraction) was extracted and stored on ice. Pellet was resuspended in 100μl of NER (nuclear extraction reagent, supplemented with protease and phosphatase inhibitors), vortexed for 15s and steps repeated every 10 min for a total of 40min. Mixture was centrifuged at full speed for 10min and extracts subjected to immunoprecipitation and immunoblotting.

**Immunoprecipitation.** Primary hepatocytes and HepG2 cells were lysed and proteins (1mg) were subjected to immunoprecipitation with an anti-menin or anti FOXO1 polyclonal antibody analyzed on SDS-PAGE, followed by immunoprobing with FOXO1 and Menin.

**Quantitative Real Time PCR.** RNA was extracted using the TRIZOL method according to the manufacturer’s protocol. Following DNAse digestion (DNAfree, Ambion), 100ng
RNA was transcribed into cDNA in a 20µl reaction using a High Capacity cDNA kit (Applied Biosystems, Carlsbad CA), analysed and amplified (ABI 7900 HT system). PCR was performed in a 10µl reaction, containing 5µl cDNA (1/5 diluted), 1x SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad CA) and 300nM of each primer.

Menin forward primer: TCATTGCTGCCCTCTATGCC

Menin reverse primer: TCCAGTTTGGTGCTGTGATG

GAPDH forward primer: CCACCAGCCCCAGCAAGAGC

GAPDH reverse primer: GGCAGGGACTCCCCAGCAGT

18s forward primer: TTGACGGAAGGGCACCACCAG

18s reverse primer: GCACCACCACCCACGGAATCG

Ct values (cycle threshold) were used to calculate the amount of amplified PCR product relative to GAPDH or 18s. The relative amount of mRNA was calculated as $2^{-\Delta CT}$.

Results were expressed as fold change as means ± SEM.

**Immunocytochemistry.** Cells were fixed in 4% paraformaldehyde/PBS on and blocked with 20% normal donkey serum/PBS and 0.1% Triton X-100 for 30 min. The slides were incubated for 1 h with a 1:50 dilution of rabbit anti-menin (Bethyl labs, Montgomery TX), insulin receptor or FOXO1 antibody. A 1:200 dilution of FITC-conjugated anti-rabbit was used as the secondary antibody.

**Insulin Radioimmunoassay.** Whole blood was collected via retro-orbital bleeding in anticoagulant treated capillary tubes at the appropriate time-points after an 18h fast and
post re-feeding. Serum was collected after whole blood was spun down at 4 degrees Celsius and the supernatant was aliquoted to new tubes and stored at -20 degrees Celsius until use. RIA was conducted using Millipore (Billerica MA) kit #SRI-13K. Briefly, 10\textsuperscript{-1} serum was pipetted into tubes in duplicate and incubated with insulin antibody overnight at 4 degrees Celsius. The next day, \textsuperscript{125}I-insulin was added to samples and samples were incubated for 24 hours at 4 degrees Celsius. Precipitating reagent was added to tubes followed by vortexing and centrifugation. All tubes were counted with a gamma counter and serum insulin was calculated from those values.

**Glucose and Insulin Tolerance Tests.** Mice were fasted for 18h (glucose) and 6h (insulin) respectively. Mice were then injected intraperitoneally with either 0.5U/kg insulin or 1.5g/kg glucose and glucose measurements were taken up to 3h post injection using Accu-Chek glucometers (Roche, Indianapolis IN).

**Statistical analysis.** Data were analyzed with SPSS software using one-factor ANOVA analysis or Student’s t-test. \( P<0.05 \) were statistically significant.

**Results:**

*Insulin Regulates Menin expression via the Insulin Receptor* - Western analysis reveals that insulin (100nM) caused a progressive decrease in menin protein levels in mouse primary hepatocytes with a pronounced effect after 24 hours (Fig. 1A).

To elucidate the mechanism by which insulin regulated menin expression, we treated stably transfected NIH-3T3 cells with human insulin receptor (3T3-hIR) and untransfected cells (3T3) (7) with 100nM insulin for 24 hours prior to evaluating menin
levels by western analysis. As shown in (Fig 1B), insulin (Ins) failed to modulate menin protein content in the native 3T3 cells. In 3T3-hIR cells, baseline menin protein level was higher than in untransfected cells, but was significantly downregulated by insulin. This observation was specific for insulin since glucagon (GLU) did not change menin expression in either cell group. The data indicate that insulin exerts a down-regulatory effect on menin expression level in a manner depending on its receptor.

Effect of multiple time points of insulin exposure on menin mRNA

We investigated the direct effect of insulin on menin in primary hepatocytes isolated from mouse liver. We determined that 100nM of insulin caused a time-dependent biphasic effect on menin expression, increasing menin mRNA at 1 hr of exposure and gradually decreasing to below baseline levels by 24hrs. The transcription initiation inhibitor, amanitin, inhibited insulin’s ability to induce menin mRNA, at 1hr to 4 hrs, however not at the 24 hour time point, indicating that insulin is an acute transcriptional activator of menin (Fig. 1C). Interestingly, while insulin had no effect on menin mRNA at 24hr hrs, the decrease in menin protein suggests a post-translational effect on expression.

Effect of LY294002 and UO126 on menin expression- Since insulin activates the PI3K/AKT signaling pathway, we investigated the effect of LY294002 on menin expression with and without insulin. LY294002 is an inhibitor of PI3K/AKT signaling that regulates cell metabolism and survival (5, 13). In Figure 2B we show that LY294002 (LY) abrogated the acute effect of insulin on menin expression between 2 and 7hrs but
not at 24hrs. Furthermore, the analysis showed that at 24 hrs both insulin and LY294002 decreased menin protein levels but together had no effect. It is interesting to note that at 24hrs AKT phosphorylation was absent with insulin treatment (Fig 2A). Figure 2B is a quantification of the near infrared signal from the conjugated secondary antibodies showing the relative protein expression of menin to GAPDH graphed from three separate experiments.

Since insulin activates the MAPK pathway we performed similar experiments with the MAPK inhibitor, UO126 and determined the effect on menin expression and ERK phosphorylation. In Figure 2C, we show that UO126 did not reverse the effect of insulin on menin expression at 2, 4 or 7h of exposure. Contrary to LY294002, UO126 effectively abrogated insulin effect on menin levels at 24hrs. Interestingly, the highest level of ERK phosphorylation is observed with insulin at 24hrs (Fig 2C), the time at which pAKT was undetectable (Fig 2A). Figure 2D is a quantification of the near infrared signal from the conjugated secondary antibodies showing the relative protein expression of menin to GAPDH graphed from three separate experiments.

*Insulin mediates Cellular Localization of menin protein* - Since AKT is known to mediate the action of insulin to sequester a number of target proteins from the nucleus to the cytoplasm (5), we hypothesized that insulin causes translocation of menin from the nucleus into the cytoplasm. Insulin treatment of primary hepatocytes caused traslocation of menin from the nucleus into the cytoplasm, as demonstrated by increased immunostaining of menin in the cytoplasmic compartment (Fig. 3A, green, middle panel). In the presence of LY294002 (LY) the effect of insulin was prevented and menin remained in the nucleus (Fig. 3A, right panel). These observations were further supported
by western analysis, which revealed a predominant menin expression in the nuclear (N) fractions in the absence of insulin (i). Insulin treatment, however, caused a reduction in menin nuclear content with a concomitant appearance in the cytoplasmic fractions (C) (ii). LY294002 prevented the negative effect of insulin on nuclear distribution of menin (iii), consistent with a role for the AKT pathway in mediating cellular localization of the menin protein. (Fig. 3B) shows the decrease in menin expression in nuclear extracts relative to Lamin B after treatment with insulin and which was abrogated with LY294002 (LY) exposure.

*Insulin enhances the interaction of menin with FOXO1* - AKT-mediated phosphorylation of FOXO 1 induces its cytoplasmic localization in response to insulin (26), thus we hypothesized that insulin induces FOXO1 and menin interaction. Co-immunoprecipitation assays detected FOXO1 in the menin immunopellet (IP) from mouse primary hepatocyte lysates treated with insulin (100nM) for 2hrs, but not 15 minutes (Fig. 4A). The level of interaction is increased by approximately 4-fold as demonstrated by the graph insert depicting FOXO1/menin expression. Co-immunoprecipitation experiments using HepG2 cells further revealed that the complex formation between menin and FOXO1 also involves 14-3-3, which mediates the translocation of FOXO1 from the nucleus into the cytoplasm (Fig. 4B). The insulin-mediated menin/14-3-3/FOXO1 complex was specific since the association of menin with other proteins implicated in insulin metabolism such as CEACAM1 (16, 17), occurred independently of insulin treatment (data not shown). We further determined the cellular localization using multiple time points at which insulin enhanced the menin FOXO1 interaction. In Figure 4C we show that insulin enhanced the cytoplasmic
interaction of menin with FOXO1 at 2hrs, after which interactions returned back to baseline by 24hrs. Furthermore, the highest reduction of menin and FOXO1 interaction in the nucleus with insulin exposure occurred at 2hrs. Figure 4D shows the relative expression of FOXO1 to total menin. Note how the interaction trends towards an increase in the cytoplasm while decreasing in the nuclei with early time points.

Since AKT is implicated in insulin regulation of FOXO1 cellular distribution, we investigated the effect of AKT inhibitor LY294002 on the insulin-mediated menin-FOXO1 interaction. As expected, insulin treatment for 2hrs, but not 15 min elevated the interaction of menin with FOXO1 by approximately 3-fold in primary hepatocytes (Fig. 5A). Interestingly, the baseline interaction of menin with FOXO1 was elevated with LY294002 in the absence of insulin, but remained unchanged with insulin treatment at 15 min and 2 hrs (Fig 5A). Interestingly, the MAPK inhibitor UO126 (UO) inhibited the interaction of menin and FOXO1 independent of insulin.

We also performed immunocytochemical analysis to demonstrate the induced translocation of menin to the cytoplasm by insulin (Fig. 5B, panel 8). Consistent with Fig. 5A, the effect of insulin was blocked by LY294002 (LY) (Fig. 5B, panel 10). Insulin caused a similar effect on FOXO1 distribution (Fig. 5B, panel 4 versus 2). Merging menin (green) and FOXO1 (red) labeling indicated a diffuse interaction between FOXO1 and menin in the presence of insulin in both the nucleus and cytoplasm (Fig. 5B, panel 14), and this interaction was localized predominately to the nucleus with LY pretreatment (Fig. 5B panel 16). Consistent with the IP data shown in Figure 5A, UO126 inhibited the interaction of menin and FOXO1 independent of insulin (panels 17 &18). Taken together, the data show that insulin sequesters menin to the cytoplasm and increases its interaction
with FOXO1 primarily via activating the AKT signaling pathway,

In order to establish a functional role for our observation in *in vitro* we first determined the physiological stimuli that will cause the interaction of menin with FOXO1. Because hepatic FOXO1 levels and activity are modulated metabolically in the liver, we subjected C57/BLK6 male mice to a fasting-refeeding paradigm prior to examining menin-FOXO1 interaction in liver lysates. Consistent with the cell data, co-immunoprecipitation analysis detected minimum FOXO1 and menin interaction in the immunopellet derived from fasted animals. However refeeding by 7 hr significantly enhanced the FOXO1 and menin interaction (Fig. 6A), in correlation with highest plasma insulin levels (Fig. 6B). Furthermore, the menin/FOXO1 interaction in the liver is present at baseline when animals are subjected to ad libitum feeding (Fig. 6A).

*Partial loss of menin in the liver increases AKT signaling and induces FOXO1 target genes.* To determine if menin had a role to play in the metabolic processes of the liver we developed a hemizygous deletion of menin specifically in hepatocytes using FVB/129S mice expressing loxP sequences on exons 3-10 of the Men1 gene crossed to C57/Blk6 mice expressing Albumin-Cre. At 6 months of age, we analyzed genes that are direct targets of FOXO1 involved in glucose synthesis. In (Fig. 7A) we show a significant decrease in menin RNA expression in mice with liver specific hemizygous deletion of menin (HET). Interestingly, while the expression of FOXO1 remained unchanged (Fig.7D), its target genes, insulin receptor (insR) (Fig. 7B), AKT (Fig 7C) as well as IGFBP1 (Fig 7E), G6Pase (Fig 7F) and PGC1α (Fig 7G) were all significantly upregulated consistent with enhanced FOXO1 activity. In contrast, the FOXO1 independent metabolic gene glucokinase (GK) was unchanged, and interestingly, the
gluconeogenic marker PEPCK also remained unchanged. Furthermore, the menin hemizygote mice displayed reduced glucose excursions during glucose tolerance test (GTT) compared with control mice while the insulin tolerance test (ITT) showed no change (Fig.8).

The implications for these findings are under investigation in our lab, however, we hypothesize that the interactions between menin and FOXO1 play a role in induction of FOXO1 specific target genes described in Fig 7 and may partially explain the observed phenotype of reduced glucose excursion in mice with partial deletion of the MEN1 gene in the liver after IPGTT but not IPITT, shown in Fig 8.

**Discussion:**

Hepatic glucose production (HGP) plays an important role in maintaining glucose homeostasis and is mediated by FOXO1, activated by fasting to induce gluconeogenic and glycogenolytic genes involved in HGP. Menin is a tumor suppressor protein that interacts and negatively regulates the activities of several transcription factors (2). Although loss of the MEN1 gene is associated with insulinomas and increased serum insulin, the reciprocal relationship between menin and insulin has not been well delineated. We show for the first time that insulin is a direct regulator of menin expression at both the transcriptional and translational levels. Furthermore insulin effect is biphasic with an initial early response mediated by AKT (Fig 2A) and by 24hrs predominately by MAPK signaling (Fig 2B).

Menin has never been previously described to be involved in FOXO1 mediated HGP, and no glucose metabolism defects have been described in liver specific menin
deficient mice. The most intriguing interpretation of our data is in support of the hypothesis that menin plays an important role in metabolism in the liver. FOXO1 has an established role in maintaining the balance between the gluconeogenesis and glycolysis pathways in times of fasting and feeding. FOXO1 localization is controlled by PKB/AKT signaling downstream of the insulin receptor. In times of feeding, insulin signaling is high, along with activation of AKT, which causes phosphorylation of FOXO1 and its chaperone, 14-3-3. This phosphorylation causes FOXO1 to be sequestered to the cytoplasm, down-regulating its transcriptional effects. In times of reduced AKT signaling (i.e. fasting), FOXO1 is nuclear and exerts its effects on various target genes such as glucose 6 phosphatase (G6Pase), which catalyzes the rate limiting step in gluconeogenesis. Our data shows that insulin enhances the interaction of menin with FOXO1 within the cytoplasm (Figs 4C & 5B panel 14) and loss of menin is associated with up-regulation of FOXO1 target genes (Fig 7). These observations suggest that menin may repress FOXO1 transcriptional activity by recruiting the FOXO1/14-3-3 complex into the cytoplasm. We propose that insulin binding to its receptor activates the AKT signaling pathway and down-regulates menin levels at early time points (acute phase), while MAPK regulation of menin sets in at later time points (chronic phase). Interestingly, the reduction in menin expression occurs at a latter time point (about 24hrs) really suggesting a MAPK mediated effect, while interaction with FOXO1 is an early time event mediated by AKT. Since menin is a rate-limiting protein required to inhibit AKT activation, its interaction directly with FOXO1 may be required to repress FOXO1 transcriptional activity by causing its translocation into the cytoplasm.

We further observed that partial deletion of menin does not affect the expression of
FOXO1 but induces the expression of FOXO1 specific genes, in support of the hypothesis that menin negatively regulates FOXO1 activity at the post-transcriptional level via direct interaction. Indeed the finding that insulin increases the interaction between menin and FOXO1 via an AKT-dependent pathway is entirely novel, and implicates menin in FOXO1 mediated effects of insulin on glucose homeostasis and production (5, 18). FOXO1 is expressed in tissues involved in energy metabolism such as the liver (25), pancreas and gut (3) and inhibition of its function by insulin (5) may require menin.

Thus our current studies suggest that while menin may be mediating the mitogenic functions of insulin, an important effect may be to modulate the insulin mediated FOXO1 regulated metabolic pathways in the liver. Furthermore, since menin is an inhibitor of AKT activation, its expression may be the rate-limiting step in maintaining the metabolic homeostasis within the liver regulated by insulin.

In summary, insulin regulates menin expression, subcellular localization and interaction with FOXO1 via nuclear-cytoplasmic shuttling. Loss of menin leads to enhanced FOXO1 activity as evidenced by specific increase in FOXO1 target genes. Although the physiological significance of insulin’s regulation on the menin/FOXO1 interaction in the liver needs to be further elucidated, this current study indicates that insulin regulation of menin expression and interaction with FOXO1 may be essential for the regulation of hepatic glucose production in the liver. We propose that menin is required for the optimal repression of FOXO1 transcriptional activity, a protein that plays crucial roles in positive and negative regulation of cell proliferation, gluconeogenesis and glycolysis.
References:


Figure Legends:

Figure 1

**Insulin regulates menin expression via the human insulin receptor (InsR).**

**A)** Immunoblot analysis of primary hepatocytes exposed to 100nM insulin for 16 and 24 hrs show a time-dependent decrease in menin protein levels.

**B)** NIH 3T3 cells stably transfected with human insulin receptors-A (3T3-hIR) were treated with 100nM insulin and 50nM glucagon for 24 hrs prior to immunoblot analysis.

**C)** mRNA analysis by RT-PCR of primary hepatocytes extracted from C57BLK/6 mice treated with 100nM insulin, or 1µg/mL of α-amanitin (sigma) prior to insulin treatment show peak expression at 1hr and 2 hrs, inhibited by amanitin at all time points. N>3 independent experiments in triplicates pooled from livers of >9 C57BLK/6 mice. Values are mean±S.E.

Figure 2

**Insulin regulates menin expression acutely via the AKT pathway followed by MAPK.**

**A)** Protein expression of menin, pAkt, Akt, and GAPDH in HepG2 lysates after overnight serum starvation was determined under the conditions of either 1h pre-treatment of LY294002 (10µM), insulin treatment (100nM), or a combination of both as indicated by the + and – signs at the allotted time-points. (LY treatment alone was collected 1h post LY administration).
B) Quantification of Western Blots from (A) by densitometry showing menin relative to GAPDH

C) Protein expression of menin, pERK, ERK, and GAPDH in HepG2 lysates after overnight serum starvation was determined under the conditions of either 1h pre-treatment of UO126 (10μM), insulin treatment (100nM), or a combination of both as indicated by + or – signs at the allotted time-points (UO treatment alone was collected 1h post UO administration).

D) Quantification of Western Blots from (C) by densitometry showing menin relative to GAPDH.

Figure 3

Insulin shuttles menin from the nucleus into the cytoplasm via AKT signaling.

A) Flourescent images of menin in HepG2 cells after 24hr exposure to 100nM insulin (INS-24hr) show cytoplasmic localization inhibited by LY294002 (INS+LY294002). (i;ii;iii) represent menin expression in cytoplasmic (C) and nuclear (N) fractionation from untreated HepG2 cells (i), HepG2 cells treated 2with 100nM insulin (ii) and HepG2 pretreated with LY294002 for 1 hr prior to insulin treatment (iii).

B) Western blotting with 30μg nuclear lysates from HepG2 cells exposed to 100nM insulin (Ins) or 10μM LY296004 1hr prior to insulin (LY+Ins). Lamin B used for control of nuclear lysates. Graph shows densitometry of near infrared band intensity from 3 experiments. **P=0.0014
Figure 4

Insulin enhances menin interaction with FOXO1 and cellular localization via AKT.

A) Immunoprecipitation with menin antibody, immunoblotted for FOXO1 shows enhanced interaction with 100nM insulin exposure at 2h and undetectable FOXO1 with 15min of insulin treatment. Graph shows immunoblot analysis quantified by near infrared fluorescence and relative ratio of FOXO1 and menin. Values are mean±S.E. of >6 experiments, ***P<0.0005.

B) Immunoprecipitation with agarose-conjugated menin antibody was immunoblotted for FOXO1 antibody, phospho and total-14-3-3 antibodies. Graphs below show Immunoblot analysis quantified by near infrared immunofluorescence and expressed as relative ratio of phospho-14-3-3 and menin or phospho-14-3-3 and total 14-3-3. Values are mean±S.E. of 3 experiments. NS=non significant.

C) Western blotting with cytoplasmic and nuclear fractionated lysates from HepG2 cells exposed to 100nM insulin at designated time-points. IP represents immunoprecipitation with menin antibody and probed for FOXO1. Input represent total FOXO1 expressed in cytoplasmic and nuclear lysates prior to IP.

D) Graphs show densitometry of near infrared band intensity from 3 experiments of relative FOXO1 to menin expression after immunoprecipitation assay (IP). **P=0.0019

Figure 5
Menin interaction with FOXO 1 is inhibited by LY294002.

A) Immunoprecipitation with menin antibody after exposure to 10μM LY294002 (LY), or 10μM UO126 1 hr prior to insulin for 15min and 2h probed with FOXO1 specific antibody and CEACAM 1 (CC1).

B) Images of HepG2 cells stained for menin (green) and FOXO1 (red) after exposure to 100nM insulin (INS), 10μM LY294002 (LY), LY294002 + insulin (LY+INS), 10μM UO126, or UO126+insulin (UO+INS). Cells were counterstained with Dapi (blue) for nuclei. Control is untreated cells (UNT).

Figure 6

Menin and FOXO 1 interaction invivo

A) C57/BLK6 mice subjected to 18h fast (F) followed by 4hr of feeding and 7hrs of refeeding. Whole liver lysates from mice were subjected to immunoprecipitation with menin antibody and immunoblotted for FOXO1 and menin by western blot analysis.

B) Plasma insulin levels of C57/BLK6 mice subjected to 18h fast (F) followed by 4hr of feeding and 7hrs of refeeding was determined by radioimmunoassay as described in materials and methods. Data analyzed using One way Anova ; Turkey post-hoc test. ***P<0.0001; **0.0017.

Figure 7
Effect of hepatic menin on FOXO1 target genes.

C57/BLK6/FVB(129S) mice hemizygous for MEN 1 in the liver were subjected to 18h fast, livers extracted and analyzed. Genes that are direct targets of FOXO1 and directly involved in hepatic glucose production shown to be differentially expressed in the liver specific hemizygous mice (HETs) vs WT based on results from quantitative real-time PCR. A) Menin mRNA is decreased in the HETs while *p<0.0145, B) insulin receptor (InsR) *p<0.0172, C) total AKT *p<0.0249, D) total FOXO1, E) IGFBP1 **p<0.0026, F) G6Pase *p<0.0030, G) PGC1α *p<0.0054, H) PEPCK, I) Glucokinase (GK).

Figure 8

Glucose metabolism in mice with liver specific hemizygote expression of menin.

A) Glucose tolerance tests (n=5/group) in liver specific hemizygote mice (Men\textsuperscript{Liv(+/−)}) and control litter mates (WT). **p<0.01, *p<0.05. B) Area under the curve of glucose tolerance test for liver specific hemizygote mice (HET) relative to same strain control litter mates (WT), *p<0.05.

C) Insulin tolerance tests (n=5/group) in liver specific hemizygote mice (Men\textsuperscript{Liv(+/−)}) and control litter mates (WT)
A. **Protein**

- 16h 24h

![Image of protein expression](image)

- *lb:* Menin
- *relb:* GAPDH

B. **Insulin signaling**

3T3 3T3-hIR

- *Ins* Glu  - *Ins* Glu

![Image of insulin signaling](image)

- *lb:* menin
- *relb:* β-actin

C. **mRNA**

![Image of mRNA expression](image)

- Menin/18S
- 0 0.5h 1h 2h 4h 24h

- *INS*
- *INS+Amanitin*
Figure 2
Figure 3

A. Untreated, INS, INS + LY

B. Menin, Laminin B

Bar graph showing the expression levels of Menin and Laminin B across different conditions: - (control), Ins (insulin), LY, and LY + Ins.
Figure 4
Figure 5

A.  \[ \text{IP (menin)} \]

\[
\begin{array}{cccccccc}
\text{INS} & - & 15\text{min} & 2h & \text{LY} & \text{LY}^{15\text{min}} & \text{LY}^{2h} & \text{UO} & \text{UO}^{15\text{min}} & \text{UO}^{2h} \\
\text{IB: FOXO (72kD)} \\
\text{relb: CC1 (110kD)} \\
\text{relb: Menin (61kD)}
\end{array}
\]

B.  

\[
\begin{array}{ccccccc}
\text{untreated} & \text{insulin} & \text{LY296004} & \text{LY+ins} & \text{UO126} & \text{UO+ins} \\
\text{FOXO} \\
\text{MENIN} \\
\text{MERGE}
\end{array}
\]
Figure 6

A.

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<th>Condition</th>
<th>FoxO1</th>
<th>Menin</th>
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<tbody>
<tr>
<td>Fasted</td>
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<tr>
<td>Refeed 4h</td>
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<tr>
<td>Refeed 7h</td>
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<tr>
<td>ad libitum</td>
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</tbody>
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B.

Plasma insulin [ng/mL]

- Fasted
- 4h refed
- 7h refed

Significance levels indicated with asterisks: *** for p < 0.001, ** for p < 0.01.
Figure 7
Figure 8

A.

B.

C.

WT RD

HET RD
Chapter 3

Menin Liver-Specific Hemizygous Mice Challenged with High Fat Diet Show Increased Weight Gain and Markers of Metabolic Impairment

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Running Title: Menin regulated phenotype during high fat feeding

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Abstract

Objective: The menin tumor suppressor protein is abundantly expressed in the liver, although no function has been identified due to lack of tumor development in Men1 null livers. We examine the phenotype of mice lacking one functional allele of Men1 (consistent with the phenotype in humans with MEN1 syndrome) challenged with high fat diet to elucidate a metabolic function for hepatic menin.

Methods: In this study, we challenged mice with a liver specific hemizygous deletion of Men1 (HETs) on a high fat diet for 3 months, and monitored the severity of metabolic changes compared to wild type (WT) counterparts fed a high fat diet. We demonstrate that the HET mice challenged with high fat diet for 3 months show increased weight gain with decreased glucose tolerance compared to WT counterparts. Along with these changes, there was a more severe serum hormone profile involving increased serum insulin, glucose, and glucagon, all hallmarks of the type 2 diabetic phenotype. In concert with increased serum hormones, we found that these mice have significantly increased liver triglycerides coupled with increased liver steatosis and inflammatory markers. Quantitative real time polymerase chain reaction (qRT-PCR) and Western Blotting studies show increases in enzymes involved with lipogenesis and hepatic glucose production.

Conclusions: We conclude that hepatic menin is required for regulation of diet induced metabolism, and our studies indicate a protective role for the Men1 gene in the liver when challenged with high fat diet.

Keywords: menin, metabolic syndrome, fatty liver, type 2 diabetes
Introduction

Menin is the product of the \textit{Men1} gene, classified as a tumor suppressor following the classical “2-Hit Hypothesis”, which involves one genetic mutation followed by a somatic mutation causing tumor formation. Loss of menin causes the multiple endocrine neoplasia type 1 (MEN1) syndrome in humans which is an autosomal dominant disorder characterized by development of neuroendocrine tumors, glucose intolerance, and type 2 diabetes\textsuperscript{1-3}. The metabolic consequences of the MEN1 syndrome have been largely ignored, and elucidating a role for menin in metabolism can be useful in identifying novel drug targets for metabolic disorders.

Recently we have shown metabolic abnormalities in mice hemizygous for menin specifically in the liver noting differences in insulin sensitivity and markers of hepatic glucose production compared to wild type (WT) littermates on normal chow\textsuperscript{4}. In the current study, we challenge menin liver-specific hemizygous mice and their wild type littermates with high fat diet for 3 months and characterized their phenotype based on metabolic parameters. Mice with hemizygous deletion of menin on a high fat diet show increasing weight gain, increases in serum hormones such as insulin and glucagon, and increases in liver triglycerides and steatosis compared to WT. The menin mice (HETs) also exhibit increases in metabolically relevant genes such as uncoupling protein 2 (UCP2), Peroxisome Proliferator-Activated receptor gamma coactivator 1-alpha (PGC-1alpha) and glucokinase (GK), all of which are associated with the up-regulation of hepatic glucose production and lipogenesis\textsuperscript{5-8}. These results demonstrate that hepatic menin plays a protective role during high fat feeding and when lost contributes to diet-induced obesity.
Materials and Methods

Animal Maintenance

Animals were kept on a 12:12-h dark-light cycle and fed Research Diets D12451 consisting of 45% calories from saturated fat, ad libitum, for 3 months starting at 3 months of age. All procedures were approved by Institutional Animal Care and Use Committee at the University of Toledo. Liver-specific menin hemizygous mice on a mix of FVB/129S mice expressing loxP sequences on exons 3–10 of the Men1 gene (Jackson Labs #005109) and C57bl/6 mice expressing Albumin-Cre (Jackson Labs #003574) were crossed, and genotyping was performed with tail lysates. Wild type (WT) and Flox (loxP) sequences with Cre expression were considered hemizygous for menin in the liver (HETs)\(^4\). All experiments were conducted with WT HFD N=7 and HET HFD N=5 unless otherwise noted.

Serum Hormone Assays and Glucose Tolerance Test

Fasting glucose was obtained using a glucometer (Accu-Chek, Roche) after mice had undergone an 18h fast. Mice were then injected intraperitoneally with 2mg/kg glucose and monitored up to 180 minutes post injection. The upper limit of detection is 600 mg/dL and glucose above that level was recorded as such. Whole venous blood was collected via the retro orbital sinus. Radioimmunoassay was then conducted to measure serum insulin (Millipore, Billerica, MA #SRI-13K), glucagon (Millipore, Billerica, MA #GL-32K), and C-peptide (Millipore, Billerica, MA #RCP-21K). The C:I ratio was calculated from the serum C-peptide and insulin values.

Triglyceride and Free Fatty Acid Measurements
Liver-specific triglyceride concentrations were determined by digesting tissue samples in chloroform-methanol (2:1 (v/v)). Briefly, the lipid layer was separated using H₂SO₄, and concentrations were determined using triglyceride assay kit (Pointe Scientific Inc, Canton, MI) and spectrophotometry. Triglycerides (TG) were normalized ng per µg of tissue. Serum triglycerides were also analyzed using Pointe Scientific Inc. triglyceride reagents. Serum free fatty acids (FFA) were calculated using NEFA C kit (Wako Chemicals, Richmond, VA).

Liver Histology

Livers were fixed in formalin and paraffin embedded. These liver sections were then stained with hemotoxylin-eosin (H&E) and assessed for the degree of steatosis and lobular inflammation according to the NASH scoring system proposed by the NIDDK-NASH Clinical Research Network⁹.

Quantitative Real-Time PCR

RNA was extracted using the 5PRIME PerfectPure RNA Tissue Kit (5 Prime, Gaithersburg, MD). RNA was transcribed into cDNA as described previously⁴. qRT-PCR was performed on the Applied Biosystems StepOnePlus system using Fast SYBR green Master Mix (Applied Biosystems, Carlsbad, CA). Probe sequences are as follows:

Menin Forward: TCATTGCTGCCCTCTATGCC

Menin Reverse: TCCAGTTTGTGCCTGTGATG

TNFα Forward: CATCTTCTAAAATTCTCGAGTGACAA
Immunoblotting Analysis

Livers were harvested and lysed in T-PER (Life Technologies, Grand Island, NY) with Complete Protease Inhibitor Mini (Roche, Indianapolis, IN) and HALT Phosphatase Inhibitor (Thermo Fisher, Waltham, MA). Protein concentration was assayed using the BCA protein assay (Pierce, Rockford, IL) and 50μg of protein was loaded per well. Samples were run on a 10% Tris Glycine gel at 125V for 2h. Blots were probed using antibodies against menin (Santa Cruz Biotechnology, Santa Cruz, CA), HNF4α (Santa Cruz Biotechnology, Santa Cruz, CA), and PGC1α (Santa Cruz Biotechnology, Santa Cruz, CA), and PGC1α (Santa Cruz Biotechnology, Santa Cruz, CA), and PGC1α (Santa Cruz Biotechnology, Santa Cruz, CA), and
Cruz, CA). Analysis was done using the Odyssey Infrared Imager with secondary antibodies conjugated to near infrared dyes (Li-Cor, Lincoln, NE).

**Statistical Analysis**

Values are reported as mean ± SEM. Student’s t test and two-way ANOVA with Bonferroni’s post test were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

\( P \) values <0.05 were considered statistically significant.

**Results**

*Menin hemizygous mice show increased weight gain and higher glucose intolerance than wild type counterparts.* Menin hemizygous mice (HETs) and their wildtype (WT) counterparts were fed high fat diet (HFD) ad libitum for a period of 3 months. As shown in Figure 1A&B, the HET mice show a “scruffy” phenotype compared to WT animals which could indicate illness or a more cytotoxic effect as a result of the high fat diet on mice deficient in menin\(^{10,11}\). During the three month period, mice were weighed initially 2 weeks post feeding, then each month after that for the duration of the experiment. As shown in Figure 1C, the HET mice showed increased weight gain compared to WT throughout the experiment, and reached statistical significance at 14 days and 60 days of high fat feeding. We performed glucose tolerance testing (IPGTT) (Figure 1D) on these mice and found that HET mice had significantly increased area under the curve (AUC) (Figure 1E), indicating increased glucose intolerance.
Menin HETs have increased serum hormones compared to WT counterparts. To further investigate the metabolic phenotype of the HET mice, serum was harvested from the mice after 3 months of high fat feeding for analysis. The HET mice showed increased levels of serum glucose, insulin, and glucagon, all consistent with a type 2 diabetic phenotype (Figure 2A-C)\textsuperscript{12,13}. To check if the increased serum insulin levels were due to increased insulin secretion from beta-cells, serum C-peptide was measured. This is a reliable indication of secretion because it is released from the beta-cell at a 1:1 ratio with insulin\textsuperscript{14}. A significant increase was observed in C-peptide indicating increased insulin secretion in these animals compared to WT (Figure 2D). Using the C-peptide and serum insulin measurements, the C:I ratio was calculated, which is a measure of insulin clearance predominantly in the liver\textsuperscript{15}. There were no significant increases in the C:I ratio in these animals (Figure 2E) indicating no problems in insulin clearance, and enforcing that the increase in serum insulin is attributed to increased insulin secretion related to insulin resistance in these animals\textsuperscript{16}.

Menin HETs show increased liver triglycerides. High fat diet is known to cause alterations of the lipid profile in mice in association with metabolic syndrome\textsuperscript{17,18}. Since serum hormones are elevated, serum triglycerides and free fatty acid (FFA) levels were examined. There was no evidence of increased serum FFA or triglycerides between the WT and HETs on high fat diet (Figure 3A&B). However, a significant increase in the level of liver tissue triglycerides was observed with no change in liver weight between the groups (Figure 3C&D). These findings indicate that loss of menin does play a protective role in the liver in the regulation of diet-induced triglyceride accumulation, consistent with a recent report using the acute hepatic knockdown of menin\textsuperscript{19}. 
Increased hepatic steatosis in HET mice compared to WT counterparts. Since the HETs exhibited increase in liver triglycerides and knowing that high fat diet contributes significantly to the development of Non-Alcoholic Fatty Liver Disease (NAFLD) which can progress to Non-Alcoholic Steatohepatitis (NASH)\textsuperscript{20,21}, livers were harvested and histologically analyzed. HET mice on high fat diet have increased liver fat droplets characterized by large size vacuoles compared to WT (Figure 4A). Using a previously described method of NASH scoring\textsuperscript{9}, it was determined that the total NASH score of the HET mice on high fat diet was significantly higher than WT counterparts on high fat diet (Figure 4B). Since NASH is associated with hepatic inflammation, the mRNA of TNFalpha in the liver of the WT and HETs on high fat diet was measured. Higher TNFalpha levels in the HET mice on HFD compared to WT were observed, signifying a significant increase in inflammation in the livers of the HET mice (Figure 4C).

HET mice show increased markers of stress, hepatic glucose production, and lipogenesis in the liver. In Figures 5A and 6A&B there are significant decreases in the mRNA and protein expression of menin in the liver of HET mice. To further investigate what contribution decrease in hepatic menin has on markers implicated in metabolic syndrome under the metabolic stress of 3 months on HFD, factors were analyzed that are involved in oxidative stress and regulation of hepatic glucose production, which have both been shown to be increased in HFD conditions\textsuperscript{17,22}. Glucokinase (GK) is a key regulatory enzyme responsible for converting glucose to glucose-6-phosphate which subsequently undergoes glycolysis and lipogenesis in the liver\textsuperscript{7,8}. GK is significantly increased in HET mice (Figure 5B), consistent with observations in diabetic patients and patients with fatty liver\textsuperscript{8}. Hepatic Nuclear Factor 4 alpha (HNF4alpha), an orphan nuclear receptor,
shows increased expression at both the mRNA and protein levels in HET mice compared to WT (Figure 5C, 6C&D). This could account for the increased expression of GK since HNF4alpha is a known positive regulator of GK expression \(^{23-26}\). UCP2, an inner mitochondrial membrane protein, has been shown to be increased in pathology such as NASH and type 2 diabetes \(^{27-29}\). A significant increase is observed in UCP2 in HET mice on high fat diet compared to WT HFD fed counterparts (Figure 5D). Since Peroxisome Proliferator-Activated receptor gamma coactivator 1-alpha (PGC1alpha) has been shown to be a regulator of UCP2 expression \(^{30}\), the mRNA and protein expression of PGC1alpha was investigated and found it to be significantly increased at the mRNA level and trending towards an increase at the protein level in HET mice compared to WT (Figure 5E, 6E&F). PGC1alpha has other well-known functions in metabolism such as regulation of factors involved in gluconeogenesis in concert with HNF4alpha \(^{26,31}\).
Discussion

The menin liver specific complete knockout has previously been shown to be void of tumors, however, no studies were undertaken on the metabolic phenotype of these mice. We have shown previously that menin hemizygous (HET) mice on normal chow do have metabolic perturbations compared to WT counterparts. When challenged with high fat diet HET mice exhibited increased weight gain compared to WT indicating that hepatic menin does have a systemic effect on the response of these mice to high fat challenge. We have also shown that these mice exhibit insulin resistance with increased glucose intolerance during glucose tolerance testing (Figure 1D). This decreased glucose tolerance is indicative of a more severe metabolic phenotype, and with the liver being a main regulator of insulin clearance, this finding therefore implicates hepatic menin as playing a role in regulating whole body glucose homeostasis. It has been previously shown that menin levels fluctuate with high fat challenge and the mice show increased hepatic steatosis. As the results confirm that finding in this study, we have expanded on this fact through a total metabolic profile showing a systemic effect of menin loss in the liver. Also, the results show a chronic effect of menin loss as opposed to acute loss, implicating menin both as an acute and chronic regulator of the metabolic state in the liver.

To further our understanding of the phenotype of these mice, we examined levels of serum hormones commonly deregulated in metabolic syndrome. Mice with prolonged exposure to HFD commonly exhibit hyperglycemia however, this consequence is exacerbated and significantly higher in the HET mice (Figure 2A). This and the increase in other serum markers indicate that HET mice are showing a decreased ability to clear
glucose from the system compared to WT mice fed high fat diet, enforcing that loss of hepatic menin affects systemic glucose homeostasis. Furthermore, the HET mice show significant increases in serum insulin concentration and increased C-peptide (Figure 2B & D) both of which are indicative of insulin resistance and increased insulin secretion due to hyperglycemia.

Glucagon secreted from the pancreas regulates hepatic glucose production (HGP) in the fasted state causing increased gluconeogenesis and glycogenolysis in the liver, and increased lipolysis in adipose tissue. Hyperglucagonemia has been implicated in hyperglycemia in models of metabolic syndrome due to its regulatory role in HGP. HET mice exhibit significantly increased levels of serum glucagon consistent with the changes observed in mice with high HGP. Previously, our lab has shown that loss of hepatic menin in mice fed normal chow have increased markers of HGP and downstream targets of FoxO1, a main regulator of the switch between fasting and feeding.

Hypertriglyceridemia is also a consequence of high fat diet that contributes to the pathogenesis of type 2 diabetes and metabolic syndrome through increased de novo production of triglycerides in the liver. Although we do not see high serum triglycerides or FFA, we do see a significant increase of triglycerides in the liver (Figure 3C) between the two groups.

Liver histology clearly shows differences in the phenotypes of HET mice compared to WT mice. WT mice fed HFD for 3 months show microsteatosis and inflammatory infiltrate, but this is to a much greater degree in HET mice on HFD (Figure 4A). The macrosteatosis and increased inflammation we see in the HET mice (Figure 4A-C) implicates menin as a protective protein for high fat diet challenge. Indeed, the
gene expression profile in HET mice challenged on HFD shows increased markers of hepatic metabolism such as glucokinase (GK), the enzyme responsible for converting glucose to glucose-6-phosphate, which is also a regulator of hepatic lipogenesis, glycolysis, and glycogen synthesis. Hepatic GK gene expression has been associated with increased hepatic lipid content, which is consistent with what we observe in our HET mice. GK gene expression is dependent on insulin signaling along with glucose concentration, but it is also regulated by factors such as HNF4alpha. As we show in this study, HNF4alpha expression is increased in HET mice on HFD (Figure 5C & 6C&D). This factor has been implicated in regulation of many genes involved in lipogenesis and HGP. It has also been cited as a co-factor working with PGC1alpha to regulate gluconeogenic genes. PGC1alpha is a master regulator of mitochondrial biogenesis, and when up-regulated its expression is associated with increased expression of protective genes such as UCP2. PGC1alpha is also a direct transcriptional regulator of UCP2, which is a mitochondrial protein responsible for inhibiting increases in membrane potential under the conditions of low adenosine diphosphate (ADP) which, in turn, decreases reactive oxygen species (ROS) production. UCP2 has been implicated in the pathogenesis of non-alcoholic steatohepatitis and non-alcoholic fatty liver disease. The phenotype of our Men1 HET mice on a high fat diet suggest that menin plays a role in mediating the expression of these important metabolically related proteins and factors described above. In conclusion, menin in the liver is a metabolic protein that functions to regulate genes involved in hepatic glucose production, lipogenesis, and steatosis in response to high fat diet.
Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References


*Endocrinology* 2006; **147**(2): 966-76.
Figure Legends

**Figure 1:** High Fat Diet (HFD) challenged HET mice show increased weight gain and glucose intolerance. A) Representative photos of WT HFD fed mice versus B) HET HFD fed mice. C) Percent change in body weight at 0, 14, 30, 60, and 90 days of high fat feeding; *p<0.05 D) Glucose tolerance test E) IPGTT AUC *p<0.0387, N=5 for both groups.

**Figure 2:** Fasting serum hormone concentrations. A) Glucose; *p<0.01 B) Insulin; **p<0.0028 C) Glucagon; *p<0.0350 D) C-Peptide; *p<0.05 E) C:I ratio

**Figure 3:** Measurement of free fatty acids (FFA) and triglycerides (TG) in HET versus WT mice on HFD. A) Serum FFA B) Serum TG C) Liver TG as ng/µg of tissue significantly increased in HET mice vs. WT; *p<0.0427 D) Liver weight as a percentage of body weight in HET vs. WT mice

**Figure 4:** Liver steatosis and increased inflammation in HET mice. Ai) Representative liver histology at 20x magnification Aii) Representative liver histology at 40x magnification B) Total NASH score; *p<0.0220 C) qRT-PCR analysis of TNFα; *p<0.0447

**Figure 5:** qRT-PCR analysis of hepatic tissue from HET and WT mice. A) Menin mRNA expression; *p<0.0484 B) Glucokinase mRNA expression; *p<0.0384 C) HNF4α mRNA expression; *p<0.0331 D) UCP2 mRNA expression; ***p<0.0001 E) PGC1α mRNA expression; *p<0.0154
Figure 6: Western Blotting Analysis of hepatic tissue from HET and WT mice. A) Menin protein B) Quantification of menin protein vs. Actin; *p<0.0205 C) HNF4α protein D) Quantification of HNF4α vs. Actin E) PGC1α protein F) Quantification of PGC1α vs. Actin
Figure 1

A) WT HFD

B) HET HFD

C) % Change in BW

D) Glucose (mg/dL)

E) IPITT AUC + SEM
Figure 2
Figure 3

(A) Serum FFA (µM)

(B) Serum TG (mg/dl)

(C) Liver TG (ng/mg)

(D) Liver Weight/BW (%)
Figure 4
Figure 5
Figure 6

A) WT HET

Menin
Actin

B) WT HET

0.00 0.05 0.10 0.15

C) WT HET

HNF4α
Actin

D) WT HET

0.0 0.5 1.0 1.5 2.0

E) WT HET

PGC1α
Actin

F) WT HET

0.0 0.1 0.2 0.3 0.4

*
Chapter 4

Discussion

4.1 Discussion

Hepatic Glucose Production (HGP) is crucial for the maintenance of normoglycemia in times of fasting. The process, gluconeogenesis, is regulated mainly by the transcription factor FoxO1. In insulin resistant and diabetic states, gluconeogenesis can continue unchecked through aberrant activation of FoxO1 leading to hyperglycemia. This demonstrates the importance of finding FoxO1 cofactors to regulate its function. Loss of the Men1 gene leads to Multiple Endocrine Neoplasia Type 1 with a phenotype of neuroendocrine tumor formation coupled with glucose intolerance and type 2 diabetes (van Wijk, Dreijerink et al. 2012). The protein product of the Men1 gene, menin, is a tumor suppressor protein that regulates many different transcription factors both positively and negatively (Balogh, Patócs et al. 2010). However, a relationship has never been shown between menin expression and the apparent metabolic manifestations of MEN1 Syndrome, we hypothesize that hepatic menin is metabolically important.

As previously mentioned, before these studies there has been no evidence linking menin and metabolic phenotypes. Menin has undergone organ specific deletions in various
mouse models to help delineate its function as a tumor suppressor (Bertolino, Tong et al. 2003; Crabtree, Scacheri et al. 2003; Chen, Yan et al. 2006), but the liver-specific knockout of menin yielded no discernible neoplastic phenotype (Scacheri, Crabtree et al. 2004). For the first time, our studies implicate insulin as a direct transcriptional and translational regulator of menin expression. Insulin mediates this effect through both major pathways downstream of the insulin receptor, with PI3K/Akt being an acute regulator of menin expression, and MAPK mediating prolonged insulin signaling effects on menin (Wuescher, Angevine et al. 2011). Not only is total protein affected after prolonged exposure to insulin, the menin protein also undergoes translocation from the nucleus to the cytoplasm. This change in subcellular localization of menin is halted by treatment with the PI3K inhibitor LY294002, showing that downstream of the insulin receptor, the PI3K/Akt pathway is mediating this effect. This change in menin localization through insulin signaling proves that menin can be regulated by important metabolic factors, but this does not exclude menin from responding to the mitogenic effects of insulin as it is a tumor suppressor protein that antagonizes growth. This led us to investigate other proteins regulated by insulin in the same manner, such as forkhead box O1 (FoxO1). As previously discussed, FoxO1 has a well studied role in the regulation of fasting and re-feeding hepatic metabolism and its localization and expression are regulated by insulin receptor signaling. Phosphorylation of FoxO1 by Akt leads to its nuclear exclusion by its chaperone protein 14-3-3 and sequestration to the cytoplasm which inhibits FoxO1 transcriptional effects (i.e. induction of gluconeogenic genes). Data shown here associates loss of menin with increases in FoxO1 target genes implicating menin as a regulator of FoxO1 function. Importantly, the gene insulin
growth factor binding protein 1 (IGFBP1), a canonical FoxO1 target indicating increased activity was upregulated, along with glucose 6 phosphatase (G6Pase), the rate limiting enzyme in gluconeogenesis (Hirota, Sakamaki et al. 2008; Haeusler, Kaestner et al. 2010). Increases in G6Pase mRNA expression in the context of FoxO1 hyperactivity has been shown to contribute to hyperglycemia in metabolic syndrome (Samuel, Choi et al. 2006; Valenti, Rametta et al. 2008). We have also shown that menin does indeed complex with FoxO1 and 14-3-3 in response to insulin signaling and believe that menin is required for continued sequestration in the cytoplasm. This effect is mediated by the PI3K/Akt pathway as co-immunoprecipitation studies show a decreased association of menin with FoxO1 at the 2 hour time-point with LY294002 treatment.

Partial loss of menin does not affect FoxO1 at the transcriptional level, showing no differences in the mRNA of FoxO1 between WT and HET animals. This observation furthers the hypothesis that menin regulates FoxO1 at the post-transcriptional level and that menin is required for insulin mediated effects on FoxO1. This data furthers our hypothesis that menin is an important metabolic protein due to its interaction with FoxO1 and mediating its transcriptional effects. The whole body effects of loss of hepatic menin are exposed through the intraperitoneal (I.P.) glucose tolerance test showing a significantly increased clearance of glucose from the periphery compared to WT counterparts. These results are further complicated by no change in the I.P. insulin tolerance test. These results indicate a whole body phenotype not necessarily dependent on whole body insulin sensitivity, leaving this effect still open to more interpretation whether it is due to increased energy expenditure or another mechanism of glucose clearance.
As we have shown an abnormal metabolic phenotype in normal chow fed mice with 50% loss of hepatic menin, we have furthered the study with high fat challenge (HFD). It has been recently shown that hepatic menin levels decrease in diabetic mouse models and diet induced obese (DIO) mice (Cheng, Yang et al. 2011). This supports our hypothesis that menin is integral in the sequestration of FoxO1 to the nucleus because it is known in genetic diabetic mouse models and DIO mouse models that FoxO1 is predominantly nuclear and exerting its effects on its target gluconeogenic genes (Gross, Wan et al. 2009; Cheng and White 2010). On HFD, HET mice show increased weight gain compared to WT counterparts again indicating a systemic effect of hepatic menin loss. Along with increased weight gain, the menin mice show an increased area under the curve (AUC) indicate decreased glucose excursion during glucose tolerance testing showing a more severe whole body metabolic phenotype than WT mice on HFD (Wuescher, Angevine et al. 2012).

To further elucidate the metabolic phenotype of these mice, we examined serum hormones commonly known to be increased in metabolic syndrome. Notably, HET mice show a significant increase in hyperglycemia compared to WT counterparts which could be exacerbated due to the mechanism described contributing to increased FoxO1 activity, which leads to increased hepatic glucose output. Serum insulin and C-Peptide are significantly increased in HET mice which indicate increased secretion secondary to hyperglycemia. The mice also show increased serum glucagon which is common in type 2 diabetes and also can exacerbate hyperglycemia (Ali and Drucker 2009). Glucagon increases hepatic glucose production (HGP) through induction of both glycogenolysis and gluconeogenesis, and can also affect adipose tissue by stimulating lipolysis (Authier
and Desbuquois 2008; Ali and Drucker 2009; Heppner, Habegger et al. 2010). HET mice have significantly increased glucagon compared to WT counterparts which is in agreement with observations in mice with high HGP.

Hypertriglyceridemia, also a well characterized manifestation of metabolic syndrome, is caused by aberrant production of triglycerides in the liver (Leavens, Easton et al. 2009; Peter, Stefan et al. 2011). Although there were no significant differences between the groups in serum triglycerides, there was indeed an increase in liver triglycerides. This was shown both through analysis of liver triglycerides and liver histology. The histology showed microsteatosis in WT mice fed HFD which was greatly exacerbated to macrosteatosis accompanied by inflammatory infiltrate in the HET mice on HFD. The non-alcoholic steatohepatitis (NASH) score of the HET mice was significantly higher than WT counterparts indicating a more severe hepatic phenotype in response to HFD. This data shows the importance of menin as protective concerning lipid accumulation in the liver due to high fat challenge. This was confirmed by another group showing loss of menin increases hepatic steatosis, however, they only conducted an acute study without further analysis of the whole body phenotype (Cheng, Yang et al. 2011). Their findings still provide insight as to the acute effects of menin loss, but importantly, our lab elaborates on a chronic whole body phenotype which is more physiologically relevant to metabolic syndrome.

Our lab had observed increases in markers of HGP in mice fed normal chow which were downstream targets of FoxO1 (Wuescher, Angevine et al. 2011) which seem to be exacerbated with HFD (Wuescher, Angevine et al. 2012). To elucidate signaling involved in the metabolic phenotypes exacerbated by high fat diet, we looked at genes
known to be deregulated in metabolic syndrome. Glucokinase (GK), the rate limiting enzyme responsible for converting glucose to glucose-6-phosphate, is associated with increased hepatic lipid content (Peter, Stefan et al. 2011) and was significantly increased in HET mice. We further examined factors also associated with increased GK expression and found both at the protein and mRNA level hepatocyte nuclear factor 4 alpha (HNF4α) was increased. HNF4α directly regulates GK expression at the transcriptional level and regulates a number of genes in concert with FoxO1. HNF4α also has been shown to be abnormally activated in hyperglycemia (Rhee, Ge et al. 2006; Oiso, Furukawa et al. 2011). Along with FoxO1, HNF4α also interacts with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) to control hepatic gluconeogenesis and lipogenesis (Yoon, Puigserver et al. 2001; Rhee, Ge et al. 2006; Estall, Kahn et al. 2009). PGC1α mRNA was increased in both normal chow fed and high fat fed HET mice compared to WT counterparts, consistent with increased FoxO1 activity and increased glucose 6 phosphatase (G6Pase) expression (Schmidt and Mandrup 2011). Along with regulation of gluconeogenic gene expression, PGC1α regulates mitochondrial biogenesis and genes responsive to oxidative stress such as uncoupling protein 2 (UCP2) (Wu, Puigserver et al. 1999). UCP2 inhibits increases in mitochondrial membrane potential during times of oxidative stress to reduce reactive oxygen species (ROS) production (Serviddio, Bellanti et al. 2008). Increases in UCP2 have been associated with non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) (Farrell and Larter 2006; Yeh and Brunt 2007; Serviddio, Bellanti et al. 2008). Mice deficient in hepatic Men1 show a metabolic syndrome phenotype which increases in severity with high fat challenge. Menin is a protective hepatic protein with
influence on whole body glucose homeostasis through regulation of FoxO1 and FoxO1 cofactors. Loss of menin does have metabolic consequences making it a candidate for drug intervention in relation to metabolic disorders.

4.2 Summary and Future Directions

The studies done here show convincing evidence of menin’s role in regulation of glucose homeostasis, but the exact mechanism has yet to be elucidated. As we have shown FoxO1 and menin do interact directly in response to insulin, it still needs to be elucidated which post-translational modifications of FoxO1 are affected. It is possible since menin seems to be integral in maintaining FoxO1 nuclear exclusion, that either phosphorylation or acetylation could be affected. During fasting and re-feeding studies, acute changes are seen in levels of insulin, glucose, and other important hormones. We started to investigate what effect acute changes will have in menin deficient livers. As shown below (Figure 4-1) we see a significant basal increase in phosphorylated Akt (pAkt) which remains unchanged with re-feeding. This indicates an abnormal response to insulin signaling and does support that menin is a negative regulator of Akt. Although not significant, we also observe an increase in acetylation of FoxO1 which could be compensatory for increased FoxO1 activity (Banks, Kim-Muller et al. 2011).

Glucokinase is differentially regulated by both hepatocyte nuclear factor 4 alpha (HNF4α) and FoxO1. HNF4α increases glucokinase (GK) expression during re-feeding while FoxO1 represses it in fasting (Hirota, Sakamaki et al. 2008), however, there is a reciprocal expression in the menin knockout livers with an increase of GK at fasting and a repression during feeding.
This decreased expression of GK in the liver at feeding, when it should be induced, could cause decreases in the downstream pathways that it effects such as glycolysis, lipogenesis, and the pentose phosphate shunt which are all crucial to post-prandial hepatic metabolism (Hagiwara, Cornu et al. 2012). This preliminary data shows that loss of menin in the liver even can have effects on acute regulation of genes involved in the
fasting/feeding responses, along with effecting post-translational modification of FoxO1. Further investigation of the metabolic phenotype of the mice is warranted as even effects are seen on normal chow fed mice. Eventual elucidation of this regulatory pathway will lead to new treatments with small molecule intervention for metabolic diseases.
References


