REGULATION OF GLUCONEOGENESIS BY A NOVEL PROTEIN PHOSPHATASE

A Dissertation

by

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ABSTRACT

Gluconeogenesis is a biochemical process through which the organs or cells can synthesize glucose from non-carbohydrate carbon substrates and is important for blood glucose homeostasis. Multiple enzymes participate in this process. Among these enzymes, PEPCK1 and G6PC are the two most important rate-limiting enzymes. Given the key roles of PEPCK1 and G6PC in gluconeogenesis, expression of these two genes is regulated by multiple transcription factors. FoxO1 and FoxO3a are the two members of FoxO family that is involved in the regulation of PEPCK1 and G6PC expressions. The subcellular localization and transcription activity of FoxO1/3a are tightly regulated by their phosphorylation status. Akt is an important regulator of FoxO1/3a through phosphorylating FoxO1/3a at the key serine/threonine residues. MAPK phosphatase 3 can promote gluconeogenesis by dephosphorylating FoxO1 in mouse liver and PP2A was reported to be a phosphatase which can dephosphorylate FoxO1/3a *in vitro* and in cells. The nuclear localized phosphatase, which can regulate FoxO1/3a function on gluconeogenesis, is not found yet. Based on microarray database, we targeted a small C-terminal phosphatase as a potential FoxOs phosphatase. The role of this phosphatase was verified by using *in vitro* dephosphorylating and binding assay, cell based biochemistry and immunofluorescence assay, and knock out mouse model. In our study, we identified SMP5 as a new FoxO1/3a phosphatase. We showed that SMP5 co-localized and interacted with FoxO1/3a in the nucleus. SMP5 dephosphorylates FoxO1/3a in ser256/253, respectively. We also found that SMP5 facilitates FoxO1/3a nuclear

localization and enhances their transcriptional activities. SMP5 KO mice have severe hypoglycemia and died within 24 hours after birth. FoxO1 and FoxO3a phosphorylation are increased. Thus, our findings indicate SMP5 is a FoxO1/3a phosphatase which controls their phosphorylation and nuclear localization, and in turn regulates their transcription activities on PEPCK1 and G6PC expression. Our study presents novel insights into the regulation of FoxO1/3a transcription activities by a new phosphatase during glucose homeostasis.

DEDICATION

To Ruifang Hua (1917-2000) and Jixiang Cai (1913-2010), my grandparents, you are always in my heart.

To Jiefu Cai and Zhengyin Cao, my parents.

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NOMENCLATURE

cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
CRTC2	CREB regulated transcription co-activator 2
CTDSP	Small C-terminal domain phosphatase
C/EBP	CCAAT-enhancer-binding protein
DAF-16	*C.elegans* forkhead box O homologue
DMEM	Dulbecco's modified eagle medium
DUSP	Dual-specificity phosphatase
EMEM	Eagle's minimum essential medium
FoxO	Forkhead box O family transcription factor
G6PC	Glucose-6-phosphatase
GST	Glutathione S-transferase
Gys1	Glycogen synthase 1
Gys2	Glycogen synthase 2
HNF4α	Hepatocyte nuclear factor 4, alpha
Het	Heterozygous
Homo	Homozygous
MEF	Mouse embryonic fibroblast
MKP3	MAP kinase phosphatase 3
NEAA	Non-essential amino acids

PEPCK1	Phosphoenolpyruvate carboxykinase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PP2A	Protein phosphatase 2
PSA	Periodic acid–Schiff staining
PTP	Protein tyrosine phosphatase
Pygl	Glycogen phosphorylase liver form
qPCR	quantitative PCR
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMP5	CTDSPL2
SMP5DN	CTDSPL2 (Asp to Asn substitution at a.a. 295)
S/TP	Serine/Threonine phosphatase
WCL	Whole cell lysate
VSD	Ventricle septum defect

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CHAPTER I

GENERAL INTRODUCTION

GLUCOSE HOMEOSTASIS

Glucose is the most important and primary energy source for the organs in human. To support the physiological function of different organs, the plasma glucose concentration should be maintained within a relatively narrow range. Neither High blood glucose (hyperglycemia) nor low blood glucose (hypoglycemia) is acceptable for body health. Long term hyperglycemia can cause diseases in different organs including: cardiovascular, kidney, nerve system, and eye. Given the limitation in the alternate fuels for the brain and the ability of glucose to be transported across the blood brain barrier, it is undoubted that blood glucose is the main energy resource for brain and hypoglycemia will cause the brain damage due to the energy supply cut off. Glucose is also the irreplaceable energy source for the mammalian erythrocytes and avian retina (Krebs 1972). The blood glucose level is sophisticate regulated during the feeding and fasting circle in mammalian. The transient increasing of blood glucose after feeding can trigger the pancreas beta cells to release insulin which in turn stimulates the glucose up take by skeletal muscle and liver. In the fasting stage, multiple hormones including glucagon, catecholamine, cortisol and growth hormone will be released into circulation and stimulate the glucose releasing by liver and kidney.

HEPATIC GLUCOSE PRODUCTION

As a major organ that plays important role in blood glucose homeostasis, liver supplies glucose to maintain the constant blood glucose level during fasting period. The glucose released from liver comes from two resources. One is glycogenolysis, through which the liver glycogen is degraded into glucose. The other one is gluconeogenesis, with this the hepatocyte can *de novo* synthesize glucose with non-carbohydrate carbon substrates (pyruvate, lactate, glycerol, glucogenic amino acids, and odd-chain fatty acids). Gluconeogenesis plays important role for blood glucose homeostasis at prolong starvation condition at which the liver glycogen is depleted by glycogenolysis.

PEPCK1 AND G6PC

There are multiple enzymes participate gluconeogenesis. Pyruvate carboxylase catalyzes the transform from pyruvate to oxaloacetate which can be transported from mitochondrial to cytoplasm. The oxaloacetate is transformed into phosphoenolpyruvate by PEPCK1. Then the phosphoenolpyruvate is transformed to glucose-6-phosphate step by step with catalysis of enolase, phosphoglycerate mutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, aldolase, fructose-1, 6 -bisphosphatse and phosphoglucose isomerase. The final step is catalyzed by glucose-6-phosphatase which transforms glucose-6-phosphate into glucose (Figure 1.1). PEPCK1 and G6PC are two rate-limiting enzymes among these enzymes for gluconeogenesis.

PEPCK1, also named as PECPK-C, is the cytosolic isoform of PEPCK and the other one is mitochondrial PEPCK which is known as PEPCK-M. PEPCK-M is the first PEPCK isoform which is found in chicken liver at 1954 (Utter and Kurahashi 1954).

PEPCK-C was found after the discovery of PEPCK-M. (Nordlie and Lardy 1963) The role of PEPCK1 in metabolism is extensively studied in the half century after it was disclosed (Chakravarty, Cassuto et al. 2005). Given the important role of PEPCK1 in glucose metabolism, it is considered to be a bio-marker for hepatic gluconeogenesis and the mRNA level of PEPCK1 is possible to be used as an indicator for the evaluation of type 2 diabetes. In adult mouse, PEPCK1 is mainly expressed in liver, kidney and adipose tissue, and it is also detected in other tissues such as skeletal muscle, brain and colon. In mouse embryo, PEPCK1 can be found in nervous system (Zimmer and Magnuson 1990).

G6PC is an endoplasmic reticulum membrane bounded phosphatase which can remove the phosphate group from glucose-6-phosphate to form glucose. The first evidence of the existing of this enzyme was reported by de Duve and co-workers at 1949, they found there is an ER cell fractionation associated phosphatase specifically acts on Glucose-6-phosphate (De Duve 1949; Hers, Berthet et al. 1951). The G6PC gene was cloned at 1993 (Lei, Shelly et al. 1993; Shelly, Lei et al. 1993) and the function of G6PC was also intensively studied after it was discovered. As an important enzyme for gluconeogenesis, G6PC mainly expresses in liver and kidney cortex(Hers and De Duve 1950). The intestine is also an organ in which G6PC expresses particularly at starvation and diabetes conditions (Rajas, Bruni et al. 1999). Furthermore, it was reported that G6PC expresses in pancreas β-cells, muscle, astrocytes and placenta (Forsyth, Bartlett et al. 1993; Khan, Hong-Lie et al. 1995; Gamberucci, Marcolongo et al. 1996; Matsubara, Takizawa et al. 1999)

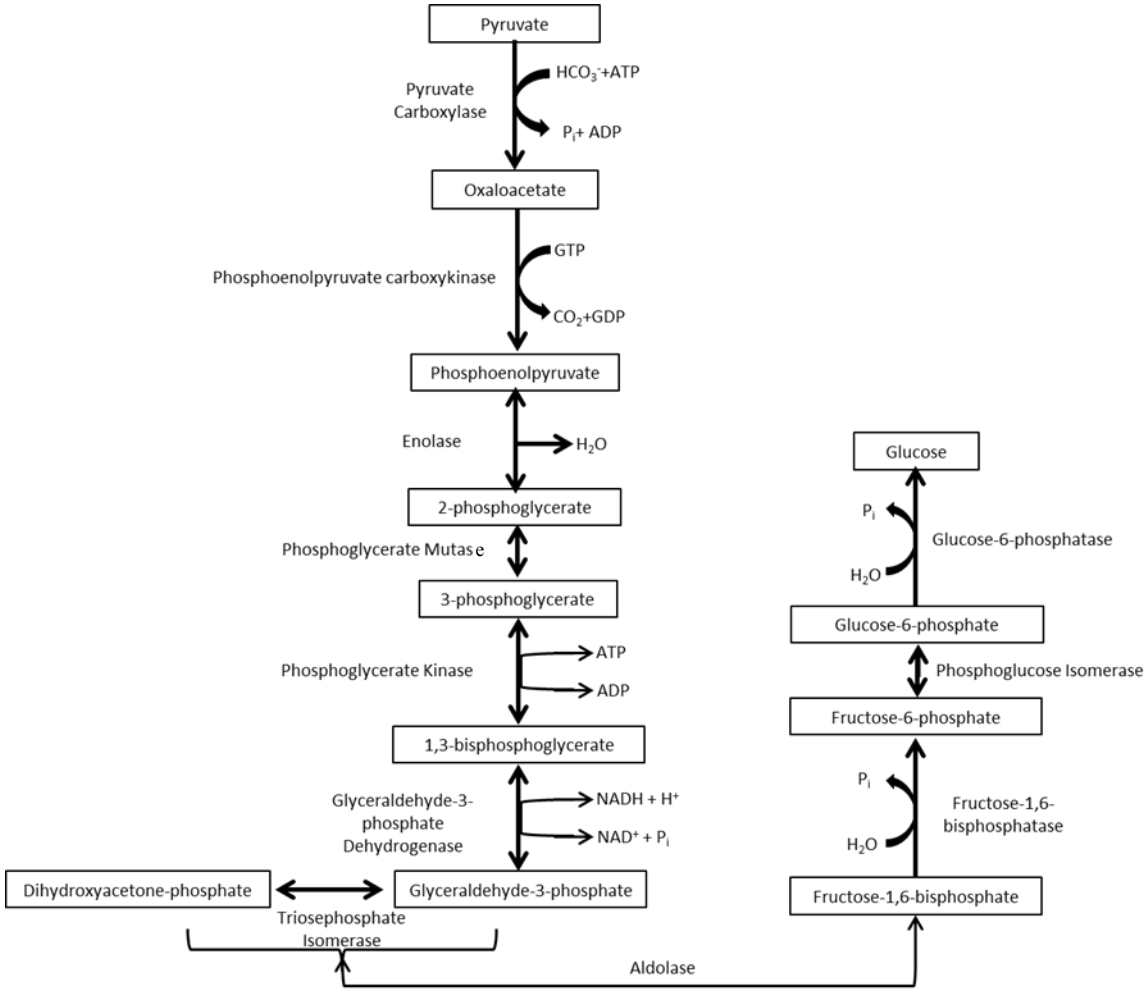


Figure 1.1 **Flow chart of gluconeogenesis.** The flow chart presents the biochemistry process of gluconeogenesis and the involved enzymes.

TRANSCRIPTIONAL REGULATION OF PEPCK1 AND G6PC IN LIVER

Consistent with the role in metabolism, the expression of PEPCK1 is tightly regulated by multiple metabolism related hormones such as insulin, glucagon, and glucocorticoid. Briefly, glucagon and glucocorticoid can stimulate PEPCK1 expression vice versa insulin can inhibit PEPCK1 expression in liver. Glucagon receptor is a G-protein coupled receptor which can activate adenylate cyclase to increase endogenous cAMP after binding with glucagon(Brubaker and Drucker 2002). As a nuclear receptor, the cytosol localized glucocorticoid receptor can re-localize into nucleus and activate down-stream genes expression including PEPCK1 and G6PC after binding with glucocorticoid (Lu, Wardell et al. 2006). There are different transcription factors and co-factors which can response to the different stimulation of the hormones and regulate PEPCK1 gene transcription. Promoter region analysis reveals multiple binding sites on the promoter for different transcription factors including glucocorticoid receptor, CREB, HNF4α, C/EBPα/β, and FoxO proteins(Chakravarty, Cassuto et al. 2005). After phosphorylated by the cAMP activated PKA, CREB binds to the cAMP-responsive element (CRE) on the PEPCK1 promoter and up-regulate PEPCK1 expression (Mayr and Montminy 2001). C/EBP can also bind to the CRE site and modulate PEPCK1 gene's transcription (Park, Roesler et al. 1990; Park, Gurney et al. 1993). The function of HNF4α on PEPCK1 expression has been proved by using HNF4α liver-specific knockout mice which has the PEPCK1 mRNA level decreasing in liver (Rhee, Inoue et al. 2003). FoxO1 can interact with the insulin responsive element (IRE) on the PEPCK1 promoter and ectopic expressing FoxO1 in *db/db* mouse liver can up-regulate PEPCK1

mRNA level (Durham, Suwanichkul et al. 1999; Altomonte, Richter et al. 2003). Besides the transcription factors, there are transcription co-factors also participate in PEPCK1 expression regulation. CREB regulated transcription co-activator 2 (CRTC2) can bind with phospho-CREB and enhance its transcription activity on PEPCK1 (Koo, Flechner et al. 2005). Peroxisome proliferator activated receptor coactivator-1 (PGC-1) can interact with HNF4α and FoxO1 to modulate PEPCK1 transcription (Rhee, Inoue et al. 2003). The transcriptional regulation of G6PC is quiet similar to the regulation of PEPCK1 given they highly related roles in the liver gluconeogenesis. CREB and its co-activator CRTC2 were found to be a positive regulator for G6PC under starvation status (Mayr and Montminy 2001; Koo, Flechner et al. 2005). Conditional knockout C/EBPα in mouse liver can dramatically decrease G6PC mRNA levels suggesting the role of C/EBPα in G6PC expression (Yang, Croniger et al. 2005). Liver G6PC expression decreasing was also found in HNF4α knockout mice (Rhee, Inoue et al. 2003). There are multiple IRE sites on the G6PC promoter on which FoxO proteins can bind and overexpression of FoxO1 can enhance G6PC expression (Schmoll, Walker et al. 2000; Vander Kooi, Streeper et al. 2003). CRTC2 and PGC-1α also were found to be the positive regulator for G6PC (Rhee, Inoue et al. 2003; Koo, Flechner et al. 2005).

FOXO PROTEIN FAMILY

Forkhead box class O (FoxO) proteins are belonging to a subgroup of the Fox family of transcription factors which have the conserved winged helix DNA-binding domain (the forkhead box). There are four FoxO genes found in mammals, including FoxO1, 3, 4 and 6. FoxO1 (FKHR) is the first member of FoxO genes and it was initially

identified in human rhabdomyosarcoma that has chromosomal rearrangements (Galili, Davis et al. 1993; Davis, D'Cruz et al. 1994). Soon after FoxO genes were found in human, the homologue of human FoxO was found in *C.elegans* as DAF-16 (Ogg, Paradis et al. 1997). It is reported that lost function mutation of daf-16 can rescue the phenotype of daf-2 (homologue of ISR) or age-1/daf23 (homologue of PI3K) mutation which suggests it play roles in insulin/PI3K/Akt signal pathway (Ogg, Paradis et al. 1997). It was shown in further studies that FoxO proteins play important roles in proliferation, metabolism, life span and stress resistance (Barthel, Schmoll et al. 2005; Greer and Brunet 2005; Kousteni 2012). Normally, FoxO proteins localize in cell nucleus in active state. In response to growth factors and insulin, FoxO proteins can be phosphorylated by Akt at three consensus Akt phosphorylation sites. Phosphorylation of FoxO proteins triggers inactivating these proteins and exporting them from nucleus (Van Der Heide, Hoekman et al. 2004).

REGULATION OF FOXO PROTEINS

As the transcription factors that active or inhibit different downstream genes which are related to different biological processes, FoxO proteins need to be tightly regulated to meet different conditions including cell proliferation, apoptosis, stress response and metabolism. FoxO are mediated in transcription/post-transcription level as well as in post-translational level. The expression of FoxO genes can be repressed by growth factors including: FGF, PDGF and IGF-1 in human fibroblast cells (Valenti, Rametta et al. 2008; Essaghir, Dif et al. 2009). FoxO3a has been reported to be directly downstream target of p53 (Renault, Thekkat et al. 2011). miR-155 has been identified as

a repressor for FoxO3a in conventional T cells and HOZOT cells (Yamamoto, Kondo et al. 2011). There are Ser/Thr sites distributing on FoxO proteins which can be phosphorylated by multiple Ser/Thr kinases (Figure 1.2 and Figure 1.3). Akt/PKB was reported to repress FoxO activity through phosphorylating FoxO and promote their nucleus exclusion (Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999).Phosphorylating by GSK is required for casein kinase1 mediated FoxO nuclear export (Rena, Woods et al. 2002). MST/Hippo can phosphorylate to disrupt their interaction with 14-3-3 and in turn facilitate nuclear localization (Lehtinen, Yuan et al. 2006; Yuan, Lehtinen et al. 2009). Cdk1 phosphorylates FoxO1 in neurons to active apoptosis (Kim and Bonni 2008; Yuan, Becker et al. 2008). AMPK was reported to phosphorylate FoxO3 and enhance its transcription activity (El Gazzar, Yoza et al. 2007). FoxO3a can be phosphorylated by Erk and its activity is repressed by the phosphorylation (Hu, Lee et al. 2004; Yang, Zong et al. 2008). Phosphatases PP2A and MKP3 dephosphorylate FoxO1 and facilitate its nuclear localization (Yan, Lavin et al. 2008; Wu, Jiao et al. 2010). Acetylation has also been found to regulate FoxO protein transcription activity. After acetylated by CBP/P300, the DNA binding capacity of FoxO3a is diminished and cytosol distribution is increased. The member of HDAC family, Sirt1 was shown to interact and deacetylate FoxO3 to affect its transcription activity (Brunet, Sweeney et al. 2004; Kobayashi, Furukawa-Hibi et al. 2005). Methylation also regulates FoxO activity, protein arginine methyltransferase PRMT1 can methylate FoxO1 and block Akt mediated phosphorylation (Yamagata, Daitoku et al. 2008). Set9 is reported to methylate FoxO3a and repress its activity (Xie, Hao et al.

2012). Finally, glycosylation was shown to be a factor which can mediate FoxO activity. With O-glycosylation, the transcription activity of FoxO1 is up-regulated and the gluconeogenesis related downstream genes expression is increased (Housley, Rodgers et al. 2008; Kuo, Zilberfarb et al. 2008).

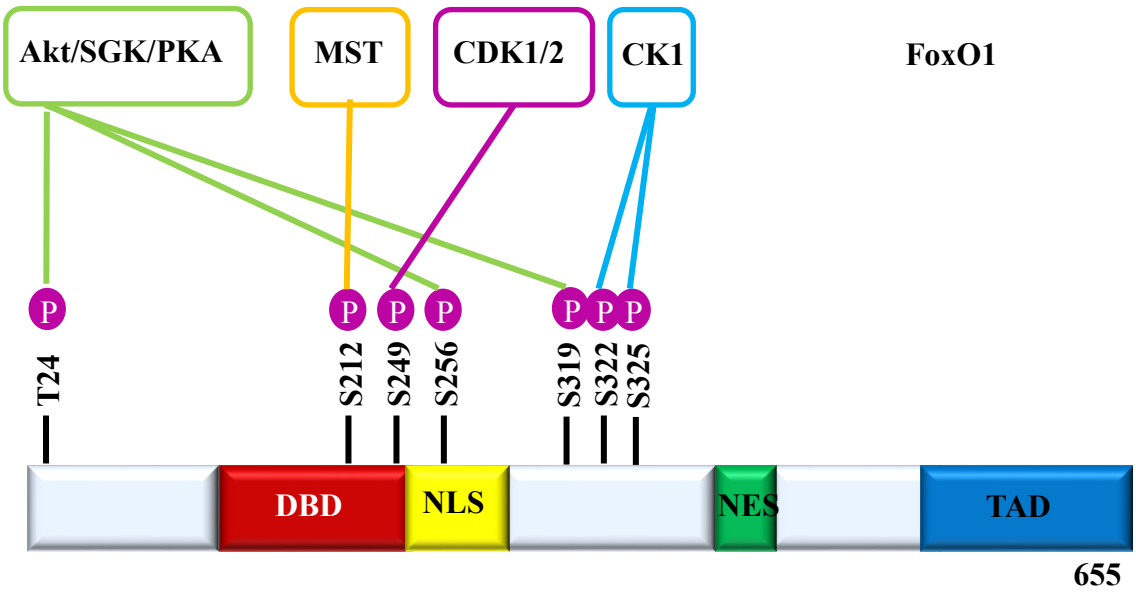


Figure 1.2 **Phosphorylation sites of human FoxO1 protein.** FoxO1 is directly phosphorylated by diverse protein kinase at multiple Serine/Threonine (S/T) sites. (1) Threonine 24 and Serine 256/319 sites are phosphorylated by Akt, SGK and PKA. (2) Serine 212 site is phosphorylated by MST. (3) Serine 249 site is phosphorylated by CDK1/2. (4) Serine 322/325 sites are phosphorylated by CK1. (DBD: DNA Binding Domain, NLS: Nucleus Localization Sequence, NES: Nucleus Export Sequence, TAD: Transcription Activation Domain)

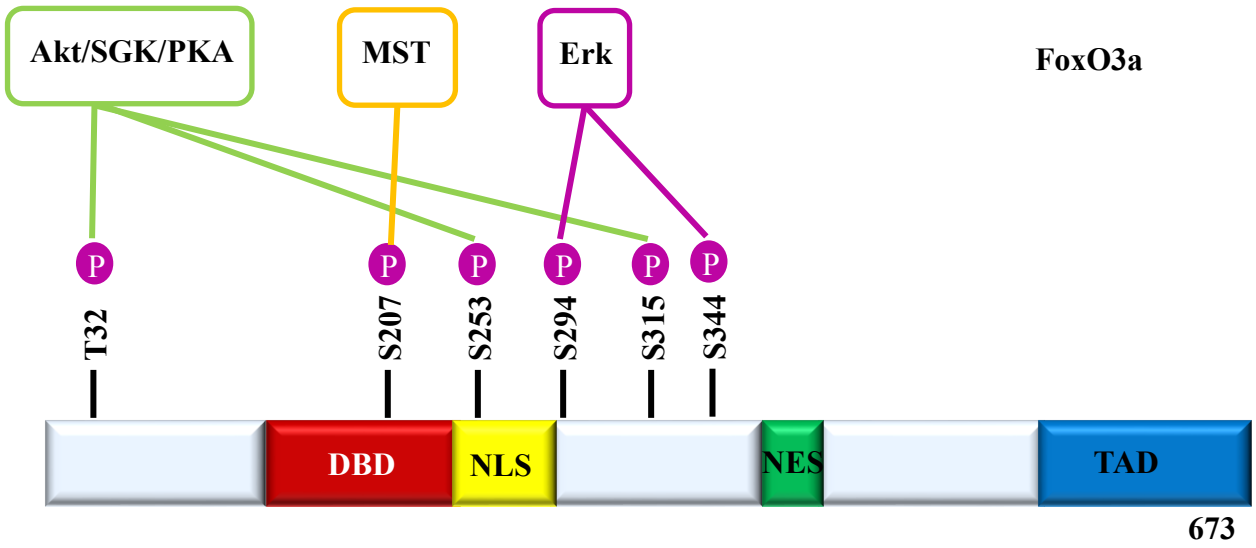


Figure 1.3 **Phosphorylation sites of human FoxO3a protein.** FoxO3a is directly phosphorylated by diverse protein kinase at multiple Serine/Threonine (S/T) sites. (1) Threonine 32 and Serine 253/315 sites are phosphorylated by Akt, SGK and PKA. (2) Serine 207 site is phosphorylated by MST. (3) Serine 294/344 sites are phosphorylated by Erk. (DBD: DNA Binding Domain, NLS: Nucleus Localization Sequence, NES: Nucleus Export Sequence, TAD: Transcription Activation Domain)

FOXO PROTEINS AND AKT

DAF-16, the *C. elegans* homologues of FoxO proteins is the first identified downstream transcriptional factor of insulin-PI3K-Akt signaling pathway (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997). As a direct upstream regulation kinase, Akt plays important role in regulating FoxO proteins transcriptional activity. Mutiple conserved Akt phosphorylation sites have been found from *C.elegans* to mammals. There are T54/24/32, S240/256/253, and S314/319/315 in DAF16, FoxO1 and FoxO3 respecively which can be phosphoralated by Akt and increasing of phosphorylation in these sites enhances the proteins nucleus export and diminishs FoxO transcriptional activity (Paradis and Ruvkun 1998; Brunet, Bonni et al. 1999; Rena, Guo et al. 1999). FoxO4 is also regulated by Akt with similar mechanism (Kops, de Ruiter et al. 1999). Among these sites, S256/253 site of is the most important and primary site for Akt regulation; Phosphoraltion of the other two sites is depend on the S256/253 site phosphorylation(Nakae, Park et al. 1999).

Akt (protein kinase B, PKB) is a serine/threonine specific protein kinase. There are three members of this family including Akt1, Akt2 and Akt3. Altough they share high similar amino acids seuqence, their functions are different. The Akt1 knockout mice have growth retardation phenotype, decreased life span under stress and increased spontaneous apoptosis in testes (Chen, Xu et al. 2001). Akt2 knockout mouse shows insulin resistance and dramatically age dependent adipose tissue decrease (Garofalo, Orena et al. 2003). The knockout mouse data suggests that Akt1 participates the singal pathways related to cell survive with the apoptosis inhibiting function and Akt2 mainly

plays a role in metabolic regulation. Unlike the ubiqutiouly expression pattern of Akt1/2, Akt3 is sepcificlly expressed in neuronal tissue and Akt3 knock out mouse has small brain size, but the function of Akt3 is not clear (Tschopp, Yang et al. 2005).

PROTEIN PHOPHATASE

As a living system, cells need to response to the changing environment and different stresses quickly and efficiently. Different signaling pathways are the ways that cell using to archive this homeostasis interaction between cells and extracellular environment. Phosphorylation and dephosphorylation of proteins play important roles in extracellular signal transducing from outside into nucleus since they are the quickest reaction for cells to response to the extracellular signals; adding and removing the phosphate group on a certain protein are used as a molecular switch to present on and off. Consistent to the important roles of phosphorylation and dephosphorylation in biophysical process, one third proteins in the total proteome have multiple phosphorylation sites which can be targeted by different kinase and phosphatase. Contrary to kinase which can phosphorylate proteins, phosphatase works as an antagonist to kinase through removing the phosphate group on target proteins added by specific kinase.

There are about 147 genes in human genome encoding protein phosphatases and these protein phosphatases are classified into three major families which include protein tyrosine phosphatases (PTPs), serine/threonine phosphatases (S/TPs) and dual-specificity protein phosphatases (DUSPs) based on the substrate preference (Barford, Das et al. 1998; Cohen 2003; Alonso, Sasin et al. 2004). In a total of 147 protein

phosphatases, there are around 38 protein tyrosine phosphatases, 40 serine/threonine phosphatases and 69 dual-specificity protein phosphatases (Barford, Das et al. 1998; Cohen 2003; Alonso, Sasin et al. 2004). Since most of the important modification sites of FoxO proteins are serine/threonine sites, serine/threonine phosphatases may play important for FoxO proteins activity regulation.

According to the amino acid sequence and catalytic domain structure, S/TPs are further categorized into three subfamilies: phosphor-protein phosphatase (PPPs), magnesium/manganese-dependent protein phosphatase (PPMs/PP2Cs), and small C-terminal domain phosphatase (FCPs) (Shi 2009). There are about 18 PPPS, 20 PPMs/PP2Cs and 7 FCPs. PPP is consisted by a highly conserved catalytic subunit associated with additional regulatory subunits which are in charge of the subcellular localization and substrate specificity of the whole enzymes. PPP can dephosphorylate different substrates and play different roles in different cells and tissues with the flexible combination of catalytic subunits and regulatory subunits. The PPMs/PP2Cs are monomeric and normally contain a conserved catalytic domain. The activity of these enzymes depends on the binding of bivalent cation (Mg^{2+} or Mn^{2+}). So far, there are 18 PPMs/PP2C family members are discovered in mammalian, they are: PPM1A (PP2Cα), PPM1B (PP2Cβ), PPM1D (wip1, PP2Cδ), PPM1E (POPX1, partner of Pix1), PPM1F (POPX2, partner of Pix2), PPM1G (PP2Cγ), PPM1H (NERRP-2C), PPM1J (PP2Cζ), PPM1K (PP2Cκ), PPM1L (PP2Cε), PPM1M (PP2Cη), PP2Cm, TA-PP2C (T-cell activation-PP2C), ILKAP (integrin-linked kinaseassociated PP2C), PHLPP1/2 (PH domain and leucine-rich repeat protein 1 and 2), and PDP1/2 (pyruvate dehydrogenase

phosphatase isoenzyme 1 and 2). Given the diverse specificity and tissue/timing expression patterns, the PPMs/PP2Cs isoforms integrate into multiple signaling pathways regulation and participate in different biophysical processes (Lammers and Lavi 2007; Stern, Privman et al. 2007; Lu and Wang 2008). The first member was found in FCP family is FCP1 which dephosphorylates small C-terminal domain of RNA polymerase II (RNAPII) at serine 2/ 5 and plays important role in RNA transcription (Cho, Kim et al. 1999; Palancade, Dubois et al. 2001). SCP1 has similar function to dephosphorylate RNAP II but it prefers to affect serine 5 (Yeo, Lin et al. 2003). Interestingly, SCP1 was reported to be a transcription repressor which can inhibit neuronal genes expression through interacting with neuron-restrictive silencer factor (Yeo, Lee et al. 2005). Further studies showed that SCP1,2,3 can dephosphorylate Smad1 and 2/3 at C-terminal and linker region to affect TGFbeta and BMP signaling pathway (Sapkota, Knockaert et al. 2006; Wrighton, Willis et al. 2006). Epithelial-mesenchyme transition (EMT) related transcription factor Snail was found to be the substrate of SCPs and dephosphorylating by SCPs can enhance Snail transcription activity in cells (Wu, Evers et al. 2009). The detailed roles and functions of SCP family members in different organs and physiological processes need to be further studied.

CHAPTER II

IDENTIFICATION OF SMP5 AS A FOXO1/3A PHOSPHATASE

INTRODUCTION

Phosphorylation and dephosphorylation is an important part of the post-transcriptional regulation of FoxO1/3a. Phosphorylating at different sites by different kinase can affect subcellular localization, protein stability and transcription activity. Among these events, Akt/PKB induced Serine256/253 phosphorylation of FxoO1/3a is the most important and primary modification for the insulin activated repression of FoxO1/3a. Phosphorylation of Serine256 is necessary for sequentially phosphorylation of Threonine24 (Rena, Prescott et al. 2001). After phosphorylated by Akt/PKB at Serine319, a consensus binding sequence is created for casein kinase 1 which can in turn phosphorylate FoxO1 at serine322 and 325 to facilitate FoxO1 cytoplasm localization (Rena, Woods et al. 2002). Given the role of Serine 256/253 phosphorylation of FoxO1/3a, it is important to identify the phosphatases which can dephosphorylate at serine 256/253 and in turn regulate transcription activity in different cell types and different physiological processes. A dual-specificity phosphatase MKP3 was reported to be a FoxO1 phosphatase which can dephosphorylate FoxO1 in cytoplasm and in turn regulate FoxO1 transcription activity on gluconeogenesis related genes. PP2A also was found to be a phosphatase of FoxO1/3a. It affects function in cell apoptosis through dephosphorylating FoxO1/3a (Yan, Lavin et al. 2008; Wu, Jiao et al. 2010). The question is raised that whether there are nuclear localized phosphatases which can

dephosphorylate and then modulate gluconeogenesis. From the diabetes database we found that the expression of SMP5, a member of small c terminal phosphatase family, is regulated during mouse metabolism profile changing (Keller, Choi et al. 2008). The expression change of SMP5 suggests that SMP5 may play roles in metabolism. We proposed that SMP5 is a potential nuclear localized FoxO proteins phosphatase. To verify the hypothesis, first of all we checked the SMP5 expression profile in wild type C57BL mice undergoing fasting and feeding cycle. The subcellular localization also be revealed by using cell fractionation and subcellular localiztion with exogenous GFP tagged SMP5 protein. To further confirm the role of SMP5 as a phosphatase for FoxO proteins, *in vivo* and *in vitro* phosphorylation assay were applied to study the SMP5 phosphatase activity.

MATERIALS AND METHODS

MOUSE

8 C57BL mice were randomly separated into 4 groups. One group was fed with regular diet feeding, one group was fasted for 12 hours, one group was fasted for 24 hours and the last group was fasted 24 hours followed with 12 hours re-feeding. After treatment, the mice were sacrificed. Mouse liver was dissected out for protein and RNA extraction. All protocols are approved by the Institutional Animal Care and Use Committee (IACUC). For protein sample, the liver was homogenized in cold RIPA buffer with protease inhibitor and centrifuged with 12,000 rpm for 10 minutes at 4°C. The supernatant was kept and the concentration was measured with Bio-Rad protein assay kit (Cat#500-0001). For RNA sample, the liver was homogenized in 1ml cold

Trizol solution and kept at room temperature for 5 minutes to complete dissociate nucleoprotein complexes. 200µl chloroform was added into tube and mixed well with shaking tube vigorously by hand for 15 seconds. The mixture was incubated at room temperature for 2 to 3 minutes. Then the sample was centrifuged with 12,000g for 15 minutes at 4°C. Supernatant was collected and 0.5ml isopropyl alcohol was added to precipitate total RNA. After spin down, the pellet was washed with 70% EtOH and re-suspended with DEPC water. The RNA concentration was measured with spectrometry.

CELL CULTURE AND TRANSFECTION

Cell culture and transfection were essentially performed as previously described (Duan et al., 2006; Lin et al., 2006). HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Cat # 11965-092) with 10% fetal bovine serum (FBS, Invitrogen, Cat # 16000-044). HepG2 cells were cultured in EMEM with 10% fetal bovine serum and NEAA. NIH3T3 cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Cat # 11965-092) with 5% fetal bovine serum (FBS, Invitrogen Cat # 16000-044). In gene expression experiments, cells were transfected using Lipofectamine LTX (Invitrogen, Cat # 15338-030) and Plus (Invitrogen, Cat # 11514-015) Reagents or Lipofectamine 2000 (Invitrogen, Cat # 11668-500) in Opti-MEM I Reduced Serum Medium (Invitrogen, Cat # 31985-062) as manufacturer's instruction (Invitrogen). For transit expression, the HeLa cells were transfected using Lipofectamin LTX and Plus and harvested 36-48 hours after transfection. For knock down stable cell line, the HepG2 cells were transfected with pRight-shSMP5-1475 and screened with 1:1000 diluted puromycin (final concentration

2 ng/mL) 48hours after transfection. Knock down efficiency was verified by Western blotting and qPCR. For immuno-staining, NIH3T3 cells were transfected with pXF-GFP-SMP5 using Lipofectamin 2000 and cells were fixed with 4%PFA for microscopy imaging.

CELL FRACTIONATION

HepG2 cells were trypsinized and washed with PBS pH7.4. The cells pellet was suspended with 300 µl buffer A ((250 mM Sucrose, 20 mM HEPES pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1mM DTT) including protease inhibitors and kept on ice for 10 minutes. The nuclear pellet was obtained by centrifuging at 1200 g for 5 minutes at 4℃ . The supernatant was collected and kept as the cytoplasmic fraction. The nuclear pellet was washed twice with 500 µl buffer A and then was suspended in buffer B (standard lysis buffer with 10% glycerol and 0.1% SDS added), lastly sonicated briefly (3 seconds) on ice at a power setting of 2-continuous.

EXPRESSION AND KNOCK DOWN PLASMIDS

HA tagged 14-3-3ζ was gotten from Dr.Dihua Yu's laboratory. Human FoxO1/3a cDNA were obtained from Dr.Qiang Tong's laboratory and were re-cloned into the pXF6F (a derivative of the CMV-driven expression vector pRK5, Genentech) vector with an N-terminal Flag tag at BamHI (5') /SalI(3') sites and BamHI(5')/HindIII(3') sites respectively. The human SMP5 wild type and point mutant (D295N) were cloned into pGTK5XF (a derivative of the GST fusion protein expression vector pGEX) and The human SMP5 wild type, DN mutant, C-terminal and N-terimnal deletion mutants were also cloned into pRK5 vector (Lin et al., 2006) with Flag, Myc, HA, GFP tag

respectively. The shRNA plasmid against mouse and human SMP5: pRight-shSMP5-1475 (target sequence GAGACAGATTTCGCTTGCATGATTT) in pRight vector (a derivative of the pSRG vector) was generated. A pair of DNA oligos (Table 2.1) was annealed with Bgl2 and Sal1 sites at two ends. The annealed double stranded oligo was cloned into linearized pRight Vector at Bgl2 and SalI sites.

DNA PRIMERS

All DNA primers (Table 2.1) were purchased from Sigma which were synthesized at a 0.2 μmol scale with the standard desalt purification.

QPCR

For each reaction, 1 μg total RNA was used as template. qScript™ cDNA SuperMix kit (Quanta Cat# 95048-100) was used for reverse transcription. 20X diluted cDNA was used to be the template for qPCR. qPCR was performed with PerfeCTa® SYBR® Green SuperMix kit (Quanta Cat# 95054-500) by using 18S as internal control.

IN VITRO PHOSPHORYLATION ASSAY

Recombinant Glutathione S-transferase (GST) fused SMP5 wild type and DN point mutant proteins were generated by purifying the bacterially expressed GST fusion proteins according to manufacturer's manual (Amersham Biosciences). Briefly, pGTK5XF-SMP5 WT/DN were transformed into BL21, the bacteria were induced by 0.5mM isopropyl-β D-thiogalactoside (IPTG) when it reach the mid-log phase (A_{600}=0.6~1.0). After 3~5 hours induction, the bacteria were harvest and lysed by GST

Table 2.1 **DNA primers and oligos sequence**

Oligo#	Name	purpose	sequence
1	qSMP5-F	qPCR	GCTGGTAGTTATGAAATGACAAAT
2	qSMP5-R	qPCR	GTTGAAGACAGGCGAAAATATGG
3	qPEPCK1-F	qPCR	TGTCGGAAGAGGACTTTGAGA
4	qPEPCK1-R	qPCR	CACATAGGGCGAGTCTGTCA
5	q18S-F	qPCR	ATTGACGGAAGGGCACCACC
6	q18S-R	qPCR	GCCAGAGTCTGTTCGTTATC
7	qGys1-F	qPCR	TTGGAAGACTGGGAGGATGA
8	qGys1-R	qPCR	CATTCATCCCCTGTCACCTT
9	qGys2-F	qPCR	GCTGAAAGACTCCCTGTGGA
10	qGys2-R	qPCR	AATTCTCTCCCCACTCATCG
11	qG6pc1-F	qPCR	TCGGAGACTGGTTCAACCTC
12	qG6pc1-R	qPCR	ACAGGTGACAGGGAACTGCT
13	qCRTC2-F	qPCR	CTTCGAGGAGGTGATGATGG
14	qCRTC2-R	qPCR	AACATTGGGCAGAGAACCAC
15	qHNF4a-F	qPCR	AAATGTGCAGGTGTTGACCA
16	qHNF4a-R	qPCR	CTCGAGGCTCCGTAGTGTTT
17	qFoxo1	qPCR	ACGAGTGGATGGTGAAGAGC
18	qFoxo1	qPCR	TGCTGTGAAGGGACAGATTG
19	qFoxo3a	qPCR	ACAAACGGCTCACTTTGTCC
20	qFoxo3a	qPCR	CTGTGCAGGGACAGGTTGT
21	qMKP3-F	qPCR	ATCACTGGAGCCAAAACCTG
22	qMKP3-R	qPCR	CATGAGGTACGCCACTGTCA
23	SMP5-a-F	In situ	GGGGTACCCCTGCCAGAGCAAAGAGGAAAT
24	SMP5-a-R	In situ	CGAGCTCGGTTCTTGGTGTTGATGTG
25	SMP5-KD-F	SMP5 KD	GATCTTCGAGACAGATTTCGCTTGCATGATTTTTGGAAATCATGCAAGCGAAATCTGTCTCTTTTTC
26	SMP5-KD-R	SMP5 KD	TCGAGAAAAAGAGACAGATTTCGCTTGCATGATTTCCAAAATCATGCAAGCGAAATCTGTCTCGAA
27	1.5K PEPCK1promoter	PEPCK1	GGGGTACCTAGCTCTTCACACAGCTTTG

Table 2.1 **Continued**

Oligo#	Name	purpose	sequence
28	1.5K PEPCK1promoter	PEPCK1	GAAGATCTATATAGAAGGGAGGACAGCC
29	1.5K G6paseprom oter	G6PC	CGACGCGTCTAGTCACATGACACCAGC
30	1.5K G6paseprom oter	G6PC	GAAGATCT TCCTCCCTTGGTACCTCAG
31	cDNA hFoxO1-F	hFoxO1c DNA	CGGGATCCTATGGCCGAGGCGCCTCAG
32	cDNA hFoxO1-R	hFoxO1c DNA	ACGCGTCGACTCAGCCTGACACCCAGCTA
33	cDNA hFoxO3a-F	hFoxO3ac DNA	CGGGATCCTATGGCAGAGGCACCGGCT
34	cDNA hFoxO3a-R	hFoxO3ac DNA	CCCAAGCTTCAGCCTGGCACCCAGCT
35	FoxO1 SA-F	FoxO1 mutation	AGAGCTGCAGCCATGGACAA
36	FoxO1 SA-R	FoxO1 mutation	TTGTCCATGGCTGCAGCTCT
37	FoxO1 SD-F	FoxO1 mutation	AGAGCTGCAGACATGGACAA
38	FoxO1 SD-R	FoxO1 mutation	TTGTCCATGTCTGCAGCTCT
39	FoxO3a SA-F	FoxO3a mutation	GGCTGTCGCCATGGACAATA
40	FoxO3a SA-R	FoxO3a mutation	TATTGTCCATGGCGACAGCC
41	FoxO3a SD-F	FoxO3a mutation	GGCTGTCGACATGGACAATA
42	FoxO3a SD-R	FoxO3a mutation	TATTGTCCATGTCGACAGCC

lysis buffer (10mM Tris-HCL pH7.5, 150mM NaCl, 1mM EDTA, 1% NP40). The GST fusion proteins were purified with Glutathione sephanose 4B beads (GE).

Flag tagged FoxO1/3a proteins were generated by using TNT *in vitro* translation kit (Promega) and purified by pulling down with Flag antibody conjugated protein A-sepharose beads. Briefly, 1μg pXF6F DNA templates were added into the TNT reaction mixture which contains TNT reaction buffer, rabbit reticulocyte lysate, T7 polymerase, amino acid mixture and RNAase inhibitor. The fully mixed reaction systems were incubated in 30°C for 1.5 hours and followed by Flag beads pull down purifying. The purified proteins were phosphorylated at serine256/253 by *in vitro* phosphorylating with purified Akt (Sigma). After phosphorylated by Akt, the Flag beads banded proteins were spun down again and washed with *in vitro* phosphatase buffer (40 mM Tris-HCl pH 8.0, 20 mM KCl and 2 mM dithiothreitol, 30 mM $MgCl_2$) to remove the Akt reaction buffer.

The phosphorylated FoxO1/3a proteins bound beads were suspended with *in vitro* phosphatase buffer and aliquoted into three Eppendorf tubes. Equal amount of GST only, GST-SMP5 WT and GST-SMP5 DN were added into the tubes and the reaction systems were incubated in 37°C for 1 hour followed by boiling with 2X SDS loading dye.

ANTIBODIES

Antibody against Flag tag was from Kodak, Myc tag (9E10, Cat#05-419) from EMD, HA tag from Babco and GFP from Invitrogen. Antibodies against pSerine256-FoxO1 (Cat# 9461) and pSerine253-FoxO3a (Cat#9466) were purchased from Cell Signaling Technology. Antibody against α-tubulin, β-actin and GAPDH were from

Sigma. Antibody against SMP5 was generated from rabbit by using N-terminal of SMP5 and validated by our laboratory.

RESULTS AND DISCUSSION

We first tested whether SMP5 expression level is changed in wild type mouse liver during fasting-feeding cycle. C57BL mice were starved for 24 hours and re-feed for 12 hours. For time zero, 12 hours fasting, 24 hours fasting and 12 hours re-feeding, 2 mice were sacrificed and the SMP5 protein and mRNA level were checked by using western blotting. Western blotting showed that during starvation the SMP5 protein increased and after re-feeding the expression of SMP5 went back to the normal level (Figure 2.1).

Next we checked the subcellular localization of SMP5. Cellular fractionation was applied to HepG2 cells. The western blotting showed that SMP5 is exclusively localized in cell nucleus (Figure 2.2). Consist to the fractionation experiment, GFP signal can only be found in the nucleus after transfecting with GFP SMP5 fusion protein in NIH3T3 cells. All the data suggests that SMP5 is a nuclear localized protein (Figure 2.3).

To investigate whether SMP5 can affect FoxO1/3a phosphorylation level in cell, pXFGFP empty vector, pXF6F SMP5 wild type and pXF6F SMP5 DN mutant were transfected into HeLa cells, 24 hours after transfection serum free DMEM medium was used to replace the regular medium for 12 hours culture. 30 minutes before harvesting the cell with 2X SDS loading dye, the serum free medium was changed to 0.2%FBS DMEM medium. The cells lysate were subjected to western blotting, α-tubulin, β-actin,

total FoxO1, total FoxO3a, phospho-FoxO1, phospho-FoxO3a, GFP and Flag tagged SMP5 were detected by using anti- α-tubulin, anti-β-actin, anti-FoxO1, anti-FoxO3a,

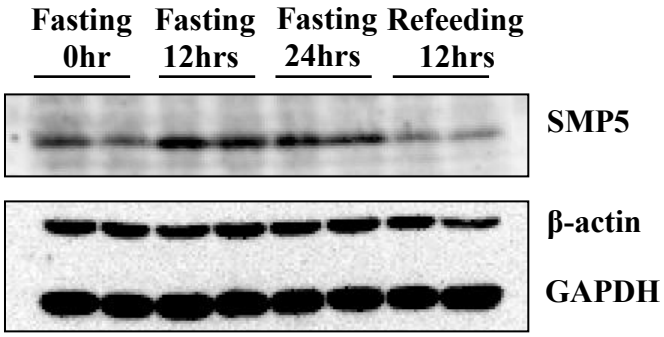


Figure 2.1 **Endogenous SMP5 protein level changes in mouse liver during fasting/feeding cycle**. C57BL mice were starved for 24 hours and re-feed for 12 hours. Mouse was sacrificed at four time points (Fasting 0 hour, Fasting 12 hours, Fasting 24 hours and Fasting 24 hours plus re-feeding 12 hours). Livers were lysed with RIPA buffer and subjected to western blotting using SMP5, β-actin and GAPDH antibodies as indicated.

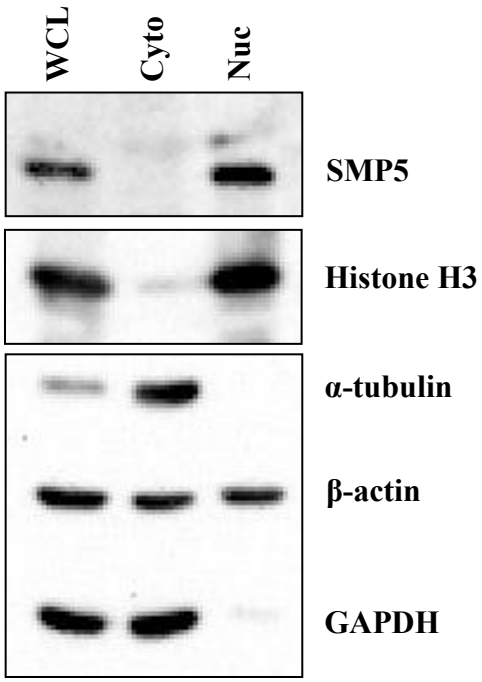


Figure 2.2 **Endogenous SMP5 locates in cell nucleus.** HepG2 cells were harvested and subjected to cellular fractionation. Whole cell lysate, cytoplasm and nuclear extraction were subjected to western blotting using SMP5, Histone3, α-tubulin, β-actin and GAPDH antibodies as indicated.

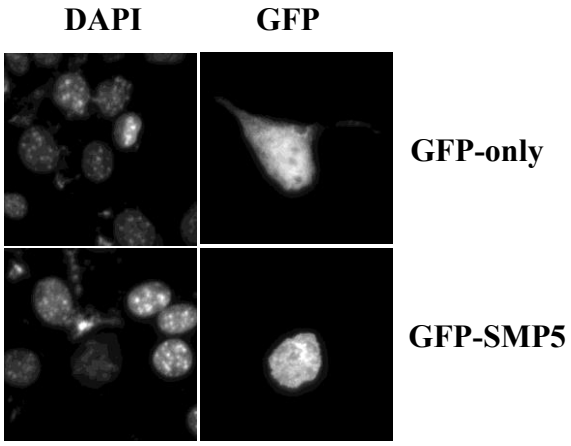


Figure 2.3 **GFP tagged SMP5 locates in nucleus of NIH3T3 cells.** GFP and GFP tagged SMP5 were transfected into NIH3T3 cells. 36 hours after transfection, cells were fixed with 4% PFA and taken pictures with fluorescence microscope. The GFP SMP5 fusion protein can only be detected in nucleus.

anti-phospho-FoxO1, anti-phospho-FoxO3a, anti-GFP and anti-Flag antibodies respectively. SMP5 knock down stable cell line was also used to verify the role of SMP5 on FoxO1/3a dephosphorylating. HepG2 SMP5 knock down and control cells were cultured in serum free EMEM medium overnight and replaced the medium to 0.2% FBS EMEM medium for 30 minutes. The cells were harvest with 2X SDS loading dye and cells lysates were subject to western blotting by using anti-α-tubulin, anti-β-actin, anti-FoxO1, anti-FoxO3a, anti-phospho-FoxO1, anti-phospho-FoxO3a and anti-SMP5 antibodies. The western blotting data showed that overexpressing SMP5 wild type can decrease phosphorylation level of endogenous FoxO1/3a but SMP5 DN mutant cannot affect it (Figure 2.4 and Figure 2.5). On the contrary, the phosphorylation level of endogenous FoxO1/3a was increased SMP5 knock down HepG2 cell line comparing to control cell line (Figure 2.6). These results suggest that SMP5 has effect on the phosphorylation level of FoxO1/3a.

Since manipulating SMP5 in cell can affect FoxO1/3a phosphorylation status, to further confirm the effect of SMP5 on FoxO1/3a is direct or indirect we studied whether SMP5 can dephosphorylate FoxO1/3a *in vitro*. Flag tagged FoxO1/3a were generated by using TNT *in vitro* translation system. After phosphorylated by commercially purchased Akt, the phosphor-FoxO1 and phospho-FoxO3a were mixed and incubated with GST-SMP5 WT and GST-SMP5 DN purified from bacteria. Reaction products were subjected to western blotting with the antibody specific for the phospho-serine256 of FoxO1 and phospho-serine253 of FoxO3a. As shown in Figure 2.7, Akt induced the phosphorylation

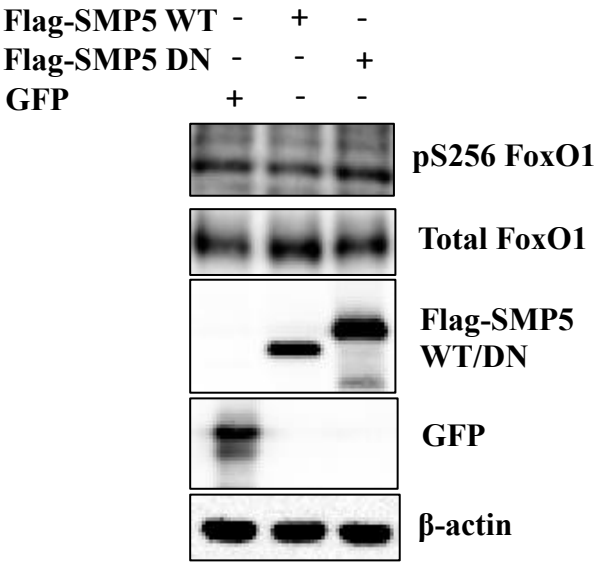


Figure 2.4 **SMP5 dephoshorylates FoxO1 in HeLa cells.** Flag tagged SMP5 wild type and DN mutant were transfected into HeLa cells. 24 hours after transfection serum free DMEM medium was used to replace the regular medium for 12 hours culture. 30 minutes before harvesting the cell with 2X SDS loading dye, the serum free medium was changed to 0.2%FBS DMEM medium. The cells lysate were subjected to western blotting using α-tubulin, β-actin, total FoxO1, phospho-FoxO1, GFP and Flag antibodies as indicated.

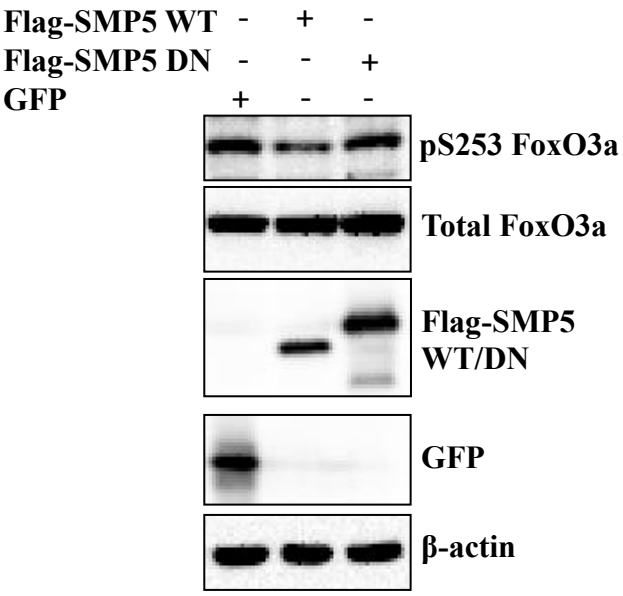


Figure 2.5 **SMP5 dephoshorylates FoxO3a in HeLa cells.** Flag tagged SMP5 wild type and DN mutant were transfected into HeLa cells. 24 hours after transfection serum free DMEM medium was used to replace the regular medium for 12 hours culture. 30 minutes before harvesting the cell with 2X SDS loading dye, the serum free medium was changed to 0.2%FBS DMEM medium. The cells lysate were subjected to western blotting using α-tubulin, β-actin, total FoxO3a, phospho-FoxO3a, GFP and Flag antibodies as indicated.

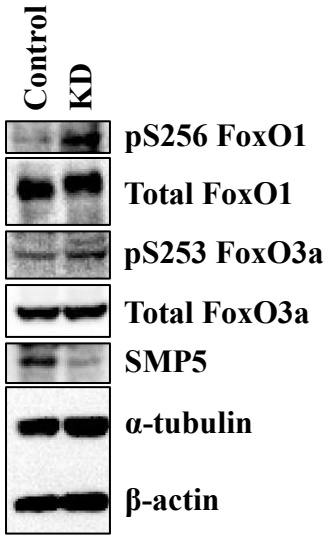


Figure 2.6 **Phosphorylation FoxO1/3a is increased in SMP5 knockdown HepG2 stable cells.** HepG2 SMP5 knockdown and control cells were cultured in serum free EMEM medium overnight and replaced the medium to 0.2% FBS EMEM medium for 30 minutes. The cells were harvest and subjected to western blotting using α-tubulin, β-actin, total FoxO1, phospho-FoxO1, total FoxO3a, phospho-FoxO3a and SMP5 antibodies as indicated.

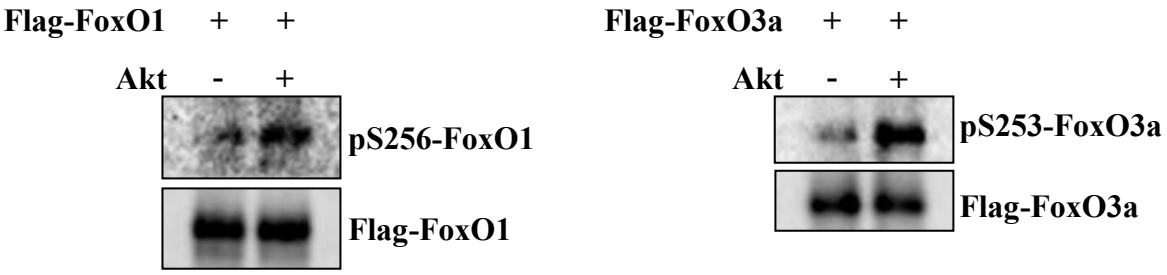


Figure 2.7 ***In vitro*** **translated FoxO1/3a is phosphorylated by Akt.** Flag tagged FoxO1/3a was translated with TNT system and pulled down with anti-Flag antibody conjugated protein A beads. Purified FoxO1/3a was incubated with Akt in Akt kinase reaction buffer and subjected to western blotting using phospho-FoxO1, phospho-FoxO3a and Flag antibodies as indicated.

of FoxO1/3a (indicated by the phospho-FoxO1 and phospho-FoxO3a level). SMP5 attenuated the Akt induced phospho-FoxO1 and phospho-FoxO3a level (Figure 2.8 and Figure 2.9). On the other hand, SMP5 DN mutant which is a phosphatase function null mutant cannot attenuate the Akt induced phospho-FoxO1 and phospho-FoxO3a level. Equal level of total Flag tagged FoxO1/3a proteins in each sample were confirmed by immune-blotting with the anti-Flag antibody. These results suggest that SMP5 can dephosphorylate FoxO1/3a directly and the effect of SMP5 on FoxO1/3a phosphorylation is related to the phosphatase activity of SMP5.

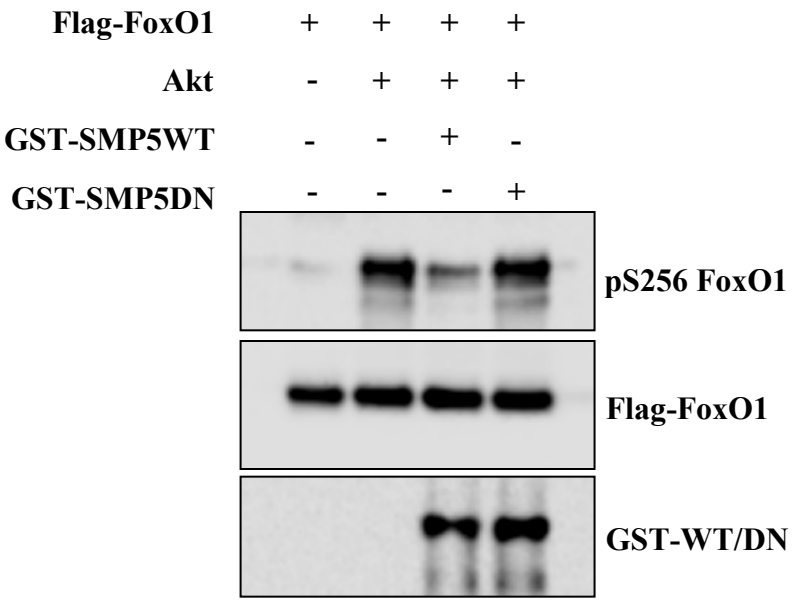


Figure 2.8 **SMP5 wild type protein directly dephosphorylates FoxO1.** Equal amount of Akt treated FoxO1 was incubated with GST, GST SMP5 fusion protein and GST SMP5 DN mutant fusion protein respectively in phosphatase reaction buffer. After incubation, products were washed and subjected to western blotting using phospho-FoxO1, Flag and GST antibodies as indicated.

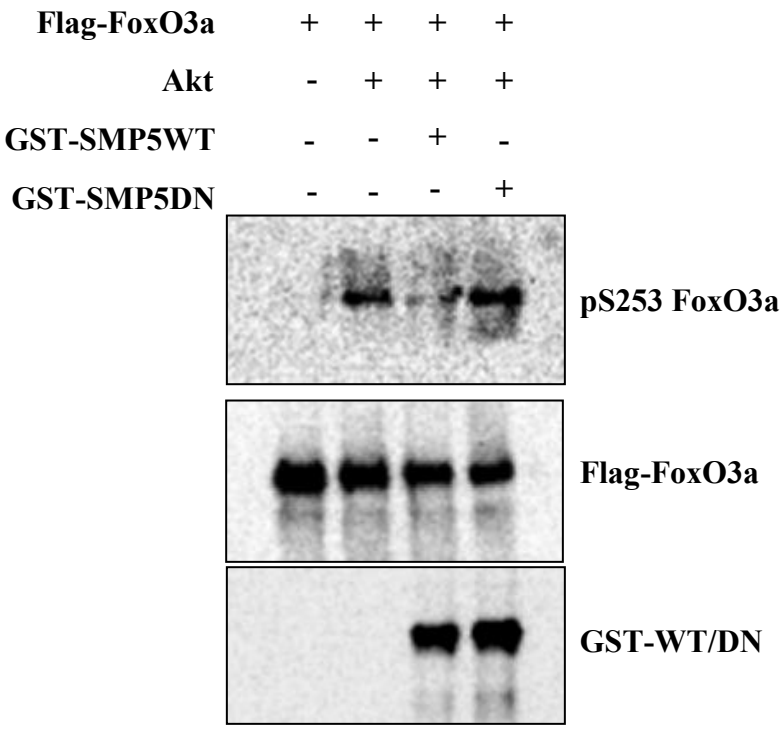


Figure 2.9 **SMP5 wild type protein directly dephosphorylates FoxO3a.** Equal amount of Akt treated FoxO3a was incubated with GST, GST SMP5 fusion protein and GST SMP5 DN mutant fusion protein respectively in phosphatase reaction buffer. After incubation, products were washed and subjected to western blotting using phospho-FoxO3a, Flag and GST antibodies as indicated.

CHAPTER III

SMP5 INTERACTS WITH FOXO1/3A *IN VITRO* AND *IN VIVO*

INTRODUCTION

Previous results suggested SMP5 can dephosphorylate in *vitro* and *in vivo*. The next question was whether SMP5 can interact with FoxO1/3a or not. To check the possibility of physical interaction between SMP5 and FoxO1/3a, Co-Immunoprecipitation and were applied. We found that SMP5 can be pulled down by using FoxO1 or FoxO3a as the bait and using anti-SMP5 antibody can also precipitate endogenous FoxO1/3a. Furthermore, the *in vitro* binding assay results showed that FoxO1/3a can bind to SMP5 directly.

MATERIALS AND METHODS

CO-IMMUNOPRECIPITATION ASSAY

HA tagged SMP5 and Flag tagged FoxO1 or FoxO3a were co-transfected into HEK293 cells. Transfected cells were harvested and lysed by 20% glycerol lysis buffer after 36 hours. The cell lysates were pre-cleaned with protein A beads and then incubated with Flag antibody conjugated protein A beads at 4°C and rotated overnight. After overnight incubation, beads were washed with Flag lysis buffer for 3 times. For endogenous CoIP, one 10mm dish 293T cells were starved with serum free medium for overnight, after starvation the cells were collected and suspended in pre chilled buffer A with protease inhibitor cocktail (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride)

and phosphatase inhibitors (NaF, Na_3VO_4)to remove the cytoplasm. The remaining nucleus was lysed in 1ml RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.25% sodium deoxycholate) with protease inhibitor cocktail (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF, Na3VO4). After pre-cleaned with 20µl protein A beads, the lysate was aliquoted into two eppendorf tubes and incubate with IgG and anti-SMP5 respectively overnight at 4°C. the antibodies associated complexes were pulled down with 20 µl protein A beads and washed with RIPA buffer for 3 times. The IP products were subjected to western blotting.

IN VITRO BINDING ASSAY

Recombinant Glutathione S-transferase (GST) fusion proteins were generated by purification of bacterially expressed GST fusion protein per manufacturer's instruction (Amersham Biosciences). *In vitro*-translated (TNT kit; Promega) Flag tagged proteins were pre-cleared with 5 µg of GST protein for 1 h then were incubated with 5 µg of different GST fusion proteins for 2 h in the in vitro binding buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 2 mM EDTA, 0.1% NP-40), with protease inhibitor cocktail (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF, Na3VO4). After extensively washed with the in vitro binding buffer proteins bound to GST fusion proteins were isolated with glutathione-Sepharose beads (Amersham Pharmacia Biotech), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the target proteins were detected with anti-Flag and anti-GST antibodies.

RESULTS AND DISCUSSION

Previous data showed that SMP5 can dephosphorylate FoxO1/3a within cells and *in vitro*. The remaining question was whether there is directly interaction between SMP5 and FoxO1/3a since a physical interaction is required for enzyme and its substrates. To answer this question, Co-immunoprecipitation was used to verify the interaction between SMP5 and FoxO1/3a. We found immunoprecipitation using anti-Flag antibody not only retrieved Flag tagged FoxO1/3a, but also HA tagged SMP5 which associated with FoxO1 or FoxO3a. As a negative control, without co-transfection of Flag tagged FoxO1 or FoxO3a, the anti-Flag antibody cannot pull down HA tagged SMP5 (Figure 3.1 and Figure 3.2). Vice versa, beside SMP5 itself, immunoprecipitation using anti-SMP5 antibody can also pull down SMP5 associated FoxO1/3a in 293T cells (Figure 3.3). CoIP data suggests there is interaction between SMP5 and FoxO1/3a in cells. To further confirm whether the interaction between SMP5 and FoxO1/3a is direct, the GST-pull down assay was used and we found that SMP5 GST fusion protein can pull down the Flag tagged FoxO1/3a, but GST protein alone cannot (Figure 3.4 and Figure 3.5). This result suggests the interactions between SMP5 and FoxO1/3a are directly.

In summary, these results indicated that SMP5 directly interact with FoxO1/3a.

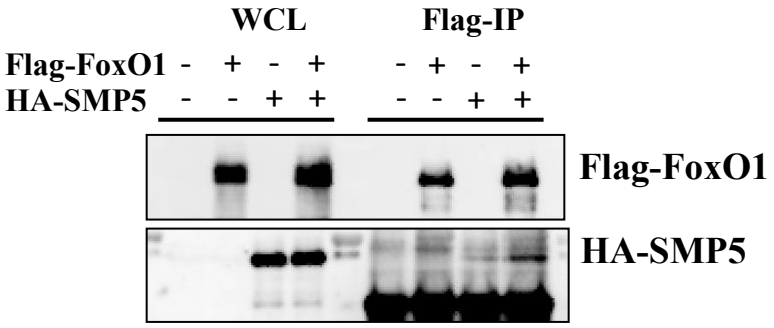


Figure 3.1 **SMP5 co-immunoprecipitates with FoxO1.** HEK293T cells were transfected with indicated expression plasmids for HA tagged SMP5 and Flag tagged FoxO1. FoxO1 or FoxO1 bound SMP5 was detected by anti-HA Western blotting analysis of the anti-Flag immunoprecipitation (IP).

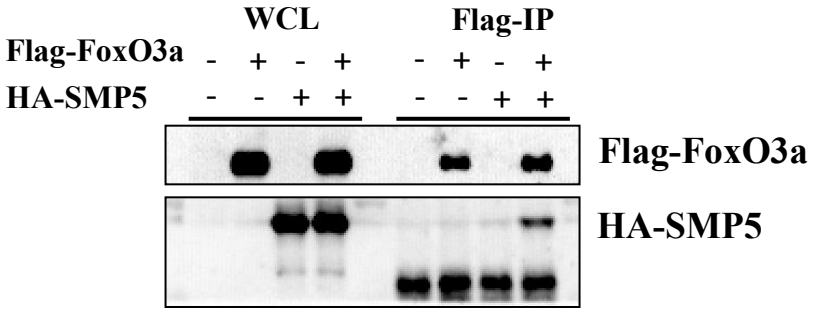


Figure 3.2 **SMP5 co-immunoprecipitates with FoxO3a.** HEK293T cells were transfected with indicated expression plasmids for HA tagged SMP5 and Flag tagged FoxO3a. FoxO3a or FoxO3a bound SMP5 was detected by anti-HA Western blotting analysis of the anti-Flag immunoprecipitation (IP).

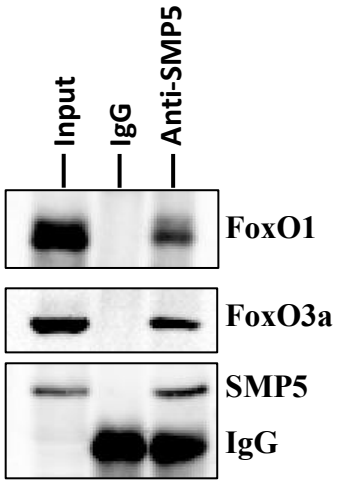


Figure 3.3 **SMP5 interacts with endogenous FoxO1/3a.** HEK 293T cells were subjected to anti-SMP5 IP. SMP5 bound FoxO1/3a were detected by anti-FoxO1 and anti-FoxO3a western blotting. Rabbit IgG was used as the control (lane 1).

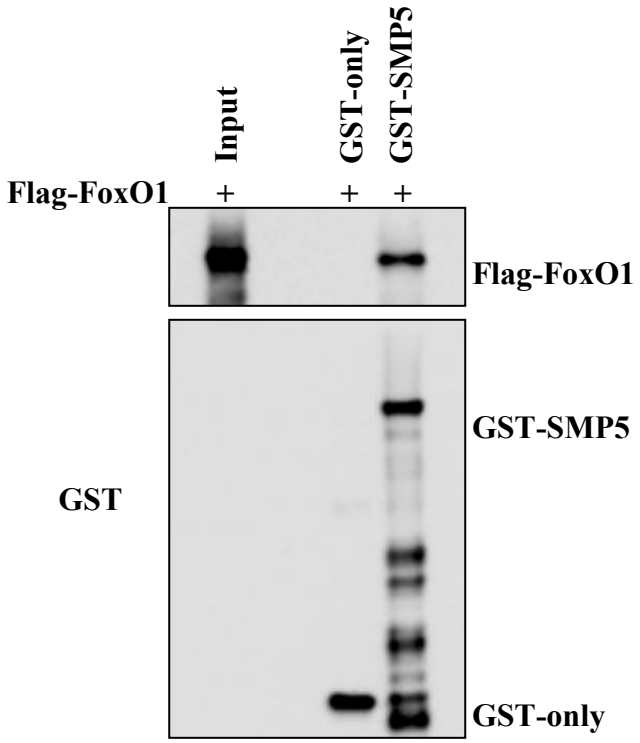


Figure 3.4 **SMP5 directly interacts with FoxO1 *in vitro*.** The pull down of *in vitro* translated Flag tagged FoxO1 by GST-SMP5, GST only as a negative control.

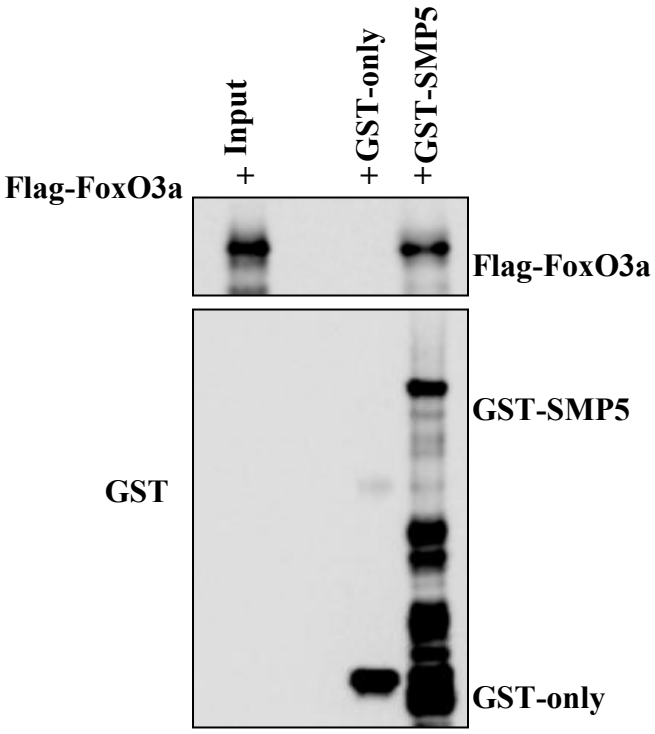


Figure 3.5 **SMP5 directly interacts with FoxO3a *in vitro*.** The pull down of *in vitro* translated Flag tagged FoxO3a by GST-SMP5, GST only as a negative control.

CHAPTER IV

SMP5 FACILITATES FOXO1/3A NUCLEUS LOCALIZATION

INTRODUCTION

Phosphorylation and dephosphorylation events play important roles in the post-transcriptional regulation of FoxO1/3a. There are multiple kinases can phosphorylate FoxO1/3a at different sites which in turn affect FoxO1/3a in different ways such as subcellular localization, protein stability and transcription activity. Among these modifications, Akt/PKB induced serine256/253 phosphorylation of is the most important and primary action for the insulin activated repression of FoxO1/3a through exporting FoxO1/3a from nucleus to cytoplasm. (A31) Since SMP5 can interact with FoxO1/3a and dephosphorylate FoxO1/3a at serine256/253 respectively, we further studied whether SMP5 can affect FoxO1/3a subcellular distribution and how it can achieve this result.

MATERIALS AND METHODS

IMMUNOSTAINING

NIH3T3 cells grew on cover slips were transfected with three plasmid combinations including Flag tagged FoxO1/3a plus GFP vector, Flag tagged FoxO1/3a plus GFP tagged SMP5 wild type and Flag tagged FoxO1/3a plus GFP tagged SMP5 DN mutant. 12 hours after transfection, the culture medium was replaced with serum free DMEM for overnight to remove the basal level insulin-Akt signal. Then, the starvation

medium was replaced by regular DMEM medium for 30 minutes. The cells were washed with PBS pH7.4 and fixed with 4%PFA. The fixed cells were washed with PBS for 3 times and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Followed permeabilization, the cells were washed again with PBS and blocked with 5% fat free milk in PBS with 0.1% Triton X-100. After blocking, the cells were blotted with 1:300 diluted mouse anti-GFP and rabbit anti-Flag antibodies at 4°C overnight. FITC conjugated anti-mouse and Texas Red conjugated anti-rabbit secondary antibodies were applied after the cells washed with PBS with 0.1% Triton X-100 for 3 times. Cover slips were mounted with DAPI contained mounting buffer and took pictures with fluorescence microscopy.

CO-IMMUNOPRECIPITATION ASSAY

Myc tagged SMP5 wild type or DN mutant were co-transfected with Flag tagged FoxO1/3a and HA tagged 14-3-3ζ. Anti-Flag antibody conjugated protein A beads were used to pull down FoxO1/3a and their associated 14-3-3 ζ as the protocol describe previously. The IP products were analyzed by western blotting with anti-Flag, anti-Myc and anti-HA antibodies.

RESULTS AND DISCUSSION

As the primary and most important phosphorylation sites of FoxO1/3a, serine 256/253 phosphorylation can repress FoxO1/3a transcription activity through inducing their nucleus export (Nakae, Park et al. 1999; Van Der Heide, Hoekman et al. 2004). Since SMP5 was found to be a potential nucleus localized phosphatase of FoxO1/3a, we first tested whether SMP5 can affect FoxO1/3a subcellular distribution. The

immunostaining data showed FoxO1/3a were transported into nucleus with serum deprivation treatment (Figure 4.1 and Figure 4.2), after replacing the serum starvation medium with regular medium, only the FoxO1/3a co-transfected with SMP5 wild type can be sustained inside the nucleus at a high ratio. Contrast to wild type SMP5 but similar to the vector control, the DN mutant did not affect the subcellular distribution of FoxO1/3a (Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4). The immunostaining data demonstrated that SMP5 can facilitate FoxO1/3a to sustain inside the nucleus. The SMP5 DN mutant failed to keep FoxO1/3a in the nucleus suggests the nucleus localization caused by SMP5 wild type co-transfection due to its phosphatase activity.

The phosphorylation induced 14-3-3ζ binding to FoxO1/3a are required for the nucleus export of FoxO1/3a (Rena, Prescott et al. 2001; Brunet, Kanai et al. 2002; Tzivion, Dobson et al. 2011). We further investigated the function of SMP5 on FoxO1/3a and 14-3-3ζ interaction by using CoIP approach. We found that the interaction between FoxO1/3a and 14-3-3ζ was disrupted when SMP5 wild type presents but not SMP5 DN mutant (Figure 4.5 and Figure 4.6). This data suggests the phosphatase activity of SMP5 is important for the function by what the interaction was affected. This disruption may due to the 14-3-3ζ masked NLS is exposed which is caused by releasing of 14-3-3ζ after dephosphorylating FoxO1/3a.

In summary, SMP5 can enhance FoxO1/3a nucleus sustaining through disrupting FoxO1/3a, 14-3-3ζ interaction.

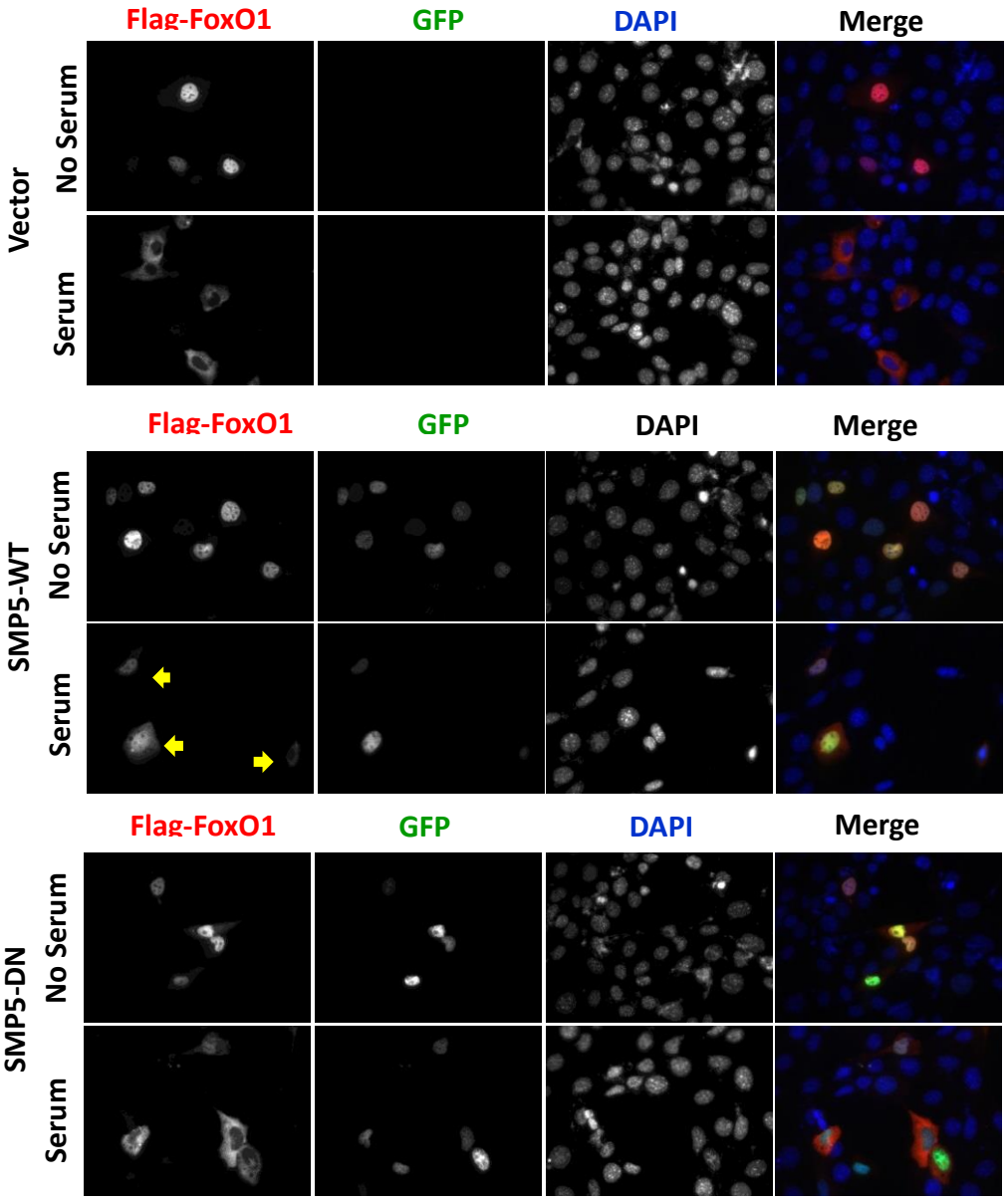


Figure 4.1 **SMP5 facilitates FoxO1 nucleus sustaining with serum treatment.** Flag tagged FoxO1 (Red) and GFP tagged SMP5 wild type or SMP5 DN mutant (Green) were co-transfected into NIH3T3 cells. Serum starved cells were treated with 5%CS, the FoxO1 with SMP5 wild type co-transfection can be detected in nucleus. (Yellow arrow)

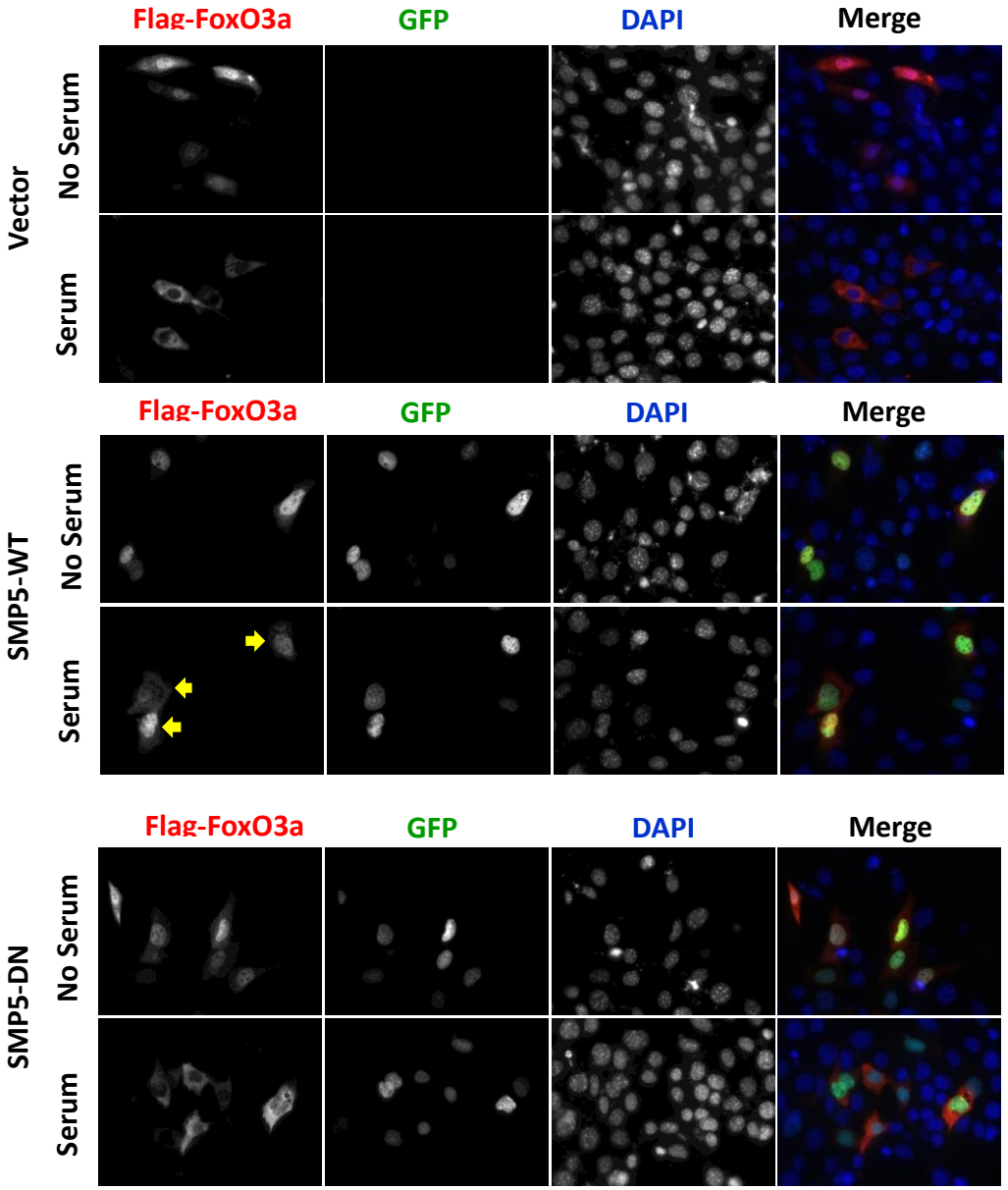


Figure 4.2 **SMP5 facilitates FoxO3a nucleus sustaining with serum treatment.** Flag tagged FoxO3a (Red) and GFP tagged SMP5 wild type or SMP5 DN mutant (Green) were co-transfected into NIH3T3 cells. Serum starved cells were treated with 5%CS, the FoxO3a with SMP5 wild type co-transfection can be detected in nucleus. (Yellow arrow)

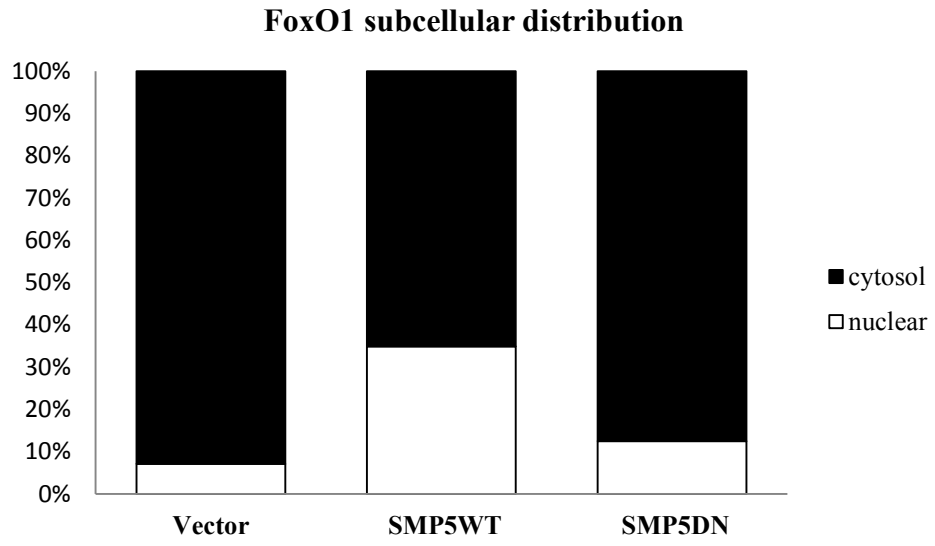


Figure 4.3 **SMP5 enhance FoxO1 nucleus sustaining.** Cell numbers with nuclear Flag staining and with cytosol Flag staining were counted in Figure 4.1. Cell numbers with nuclear Flag signal is increased with SMP5 co-transfection.

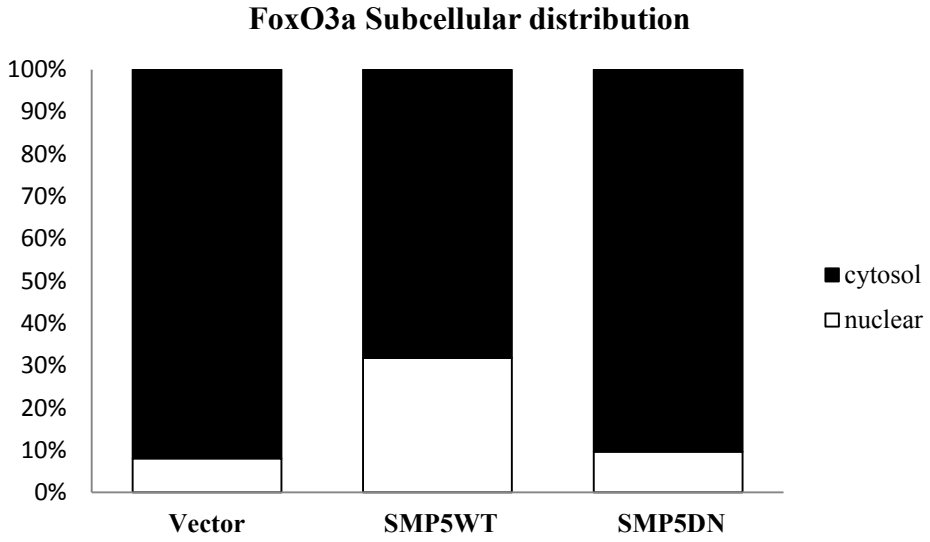


Figure 4.4 **SMP5 enhance FoxO3a nucleus sustaining.** Cell numbers with nuclear Flag staining and with cytosol Flag staining were counted in Figure 4.2. Cell numbers with nuclear Flag signal is increased with SMP5 co-transfection.

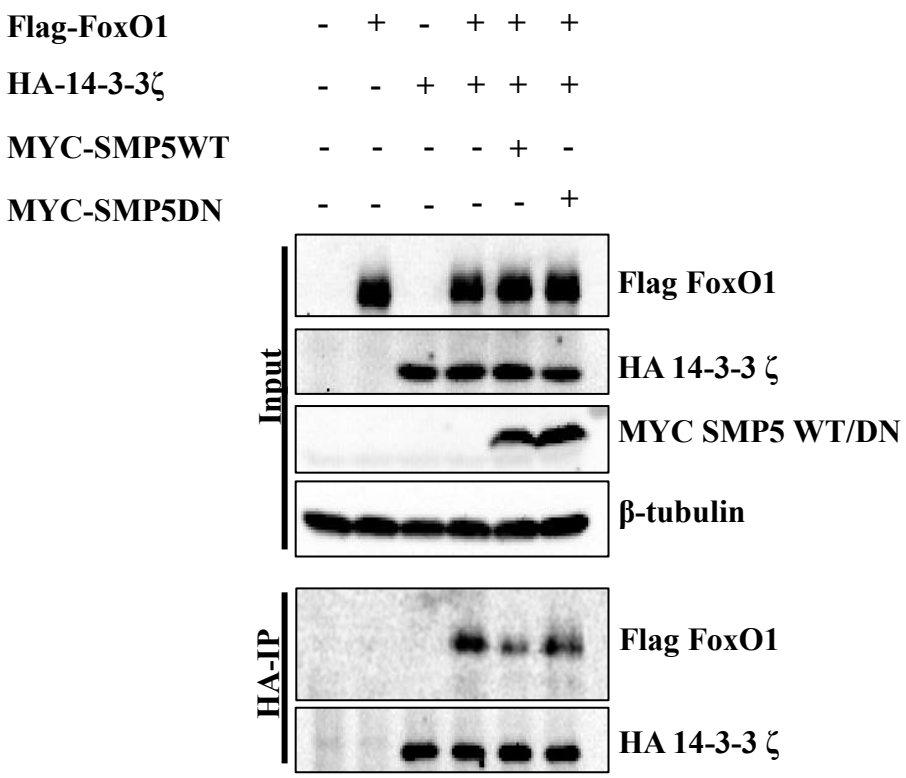


Figure 4.5 **SMP5 wild type, but not SMP5 DN mutant, affects FoxO1-14-3-3 ζ association.** HEK293T cells were transfected with expression plasmids for Flagged tagged FoxO1, HA tagged 14-3-3 ζ and MYC tagged SMP5 WT/DN. 14-3-3 ζ bound FoxO1 was detected by anti-HA IP and anti-Flag western blotting.

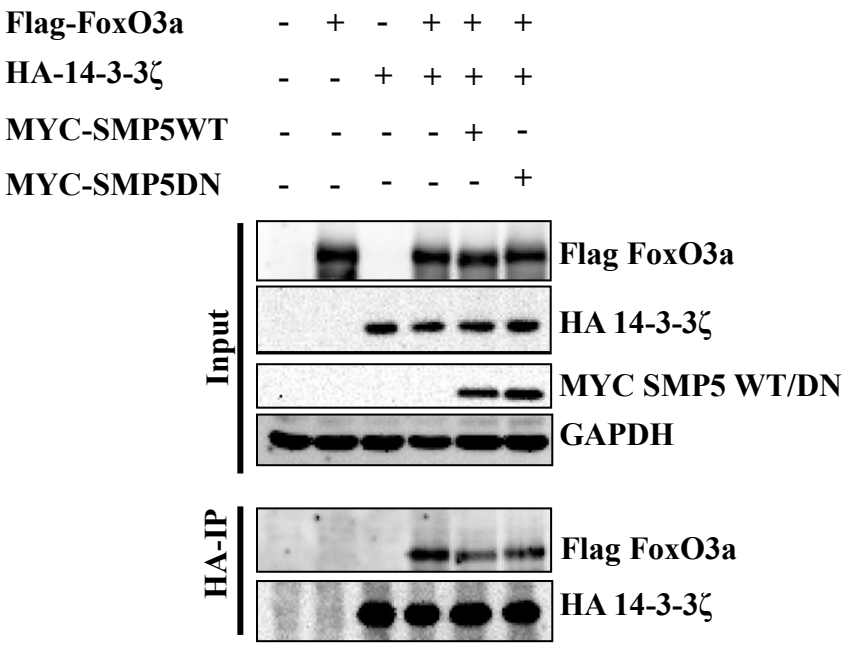


Figure 4.6 **SMP5 wild type, but not SMP5 DN mutant, affects FoxO3a-14-3-3 ζ association.** HEK293T cells were transfected with expression plasmids for Flagged tagged FoxO3a, HA tagged 14-3-3 ζ and MYC tagged SMP5 WT/DN. 14-3-3 ζ bound FoxO3a was detected by anti-HA IP and anti-Flag western blotting.

CHAPTER V

SMP5 ENHANCES FOXO1/3A TRANSCRIPTION ACTIVITY

INTRODUCTION

Phosphorylation and nucleus export of FoxO1/3a induced by Akt is the major process through which insulin inhibits FoxO1/3a transcription activity on gluconeogenesis related genes. (Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999) SMP5 is shown to be the phosphatase of FoxO1/3a and it can facilitate FoxO1/3a nucleus localization. We next investigate whether SMP5 can affect FoxO1/3a transcription activity on gluconeogenesis related genes. Two key downstream genes of FoxO1/3a, PEPCK1 and G6PC, were used to be our targets for the FoxO1/3a transcription activity study. To explore the potential of SMP5's ability to affect FoxO1/3a transcription activity, luciferase reporter assay and glucose production assay approaches were applied.

MATERIALS AND METHODS

LUCIFERASE REPORTER ASSAY

1.5kb mouse PEPCK1 promoter sequence was amplified from MEF cells genomic DNA extraction with Pfu high fidelity Taq (Promega) and cloned into pGL3-basic luciferase reporter assay vector digested with KpnI and BglII. 1.5kb mouse G6PC promoter sequence was amplified from MEF cells genomic DNA extraction with Pfu high fidelity tag (Promega) and cloned into pGL3-basic luciferase reporter assay vector

digested with MluI and BglII. Different combination including PEPCK1/G6PC reporter alone, PEPCK1/G6PC reporter with FoxO1/3a, and PEPCK1/G6PC reporter with FoxO1/3a and SMP5 wild or DN mutant were transfected into NIH3T3 cells with lipofectamin 2000. pSV-β-gal (Promega), which expresses β-galactosidase driven by the simian virus 40 early promoter, was co-transfected as the transfection efficiency indicator used to normalize the luciferase activity reading. The amount of luciferase reporter plasmid DNAs used for transfection was 75ng/well and pSV-βgal was 10ng/well (in a 12-well plate), addition vector DNA was add to make the total amounts of transfected DNA equal. 36 hours after transfection, cells were harvested and lysed with luciferase reporter lysis buffer (Promega). Centrifuging the lysates with 12,000rpm at 4°C for 10 minutes and measuring the luciferase and β-galactosidase activities by using reporter assay kit (Promega). All data sets were done with duplication and all values were normalized with β-galactosidase activities for transfection efficiency.

GLUCOSE PRODUCTION ASSAY

SMP5 knock down HepG2 stable and scramble control were used for glucose production assay. Briefly, 0.6 million HepG2 cells were seed in 12 wells plate one day before treatment. The day after cells seeding, 600μl glucose production medium (no glucose, no phenol red and serum free DMEM containing gluconeogenic substrates, 20mM sodium lactate, and 2mM sodium pyruvate) was used to replace the regular medium with after 3 times wash with warm PBS and the cells were incubated for 13 hours. Glucagon was added into the glucose production medium with the final concentration 100mM for extra 3 hours treatment. The medium was collected to measure

the glucose concentration with Amplex® Red Glucose/Glucose Oxidase Assay Kit (Invitrogen Cat# A22189) according to the product manual. The cells were lysed with RIPA for protein concentration measurement. The glucose concentration was normalized by protein concentration.

RESULTS AND DISCUSSION

PEPCK1 and G6PC are two key enzymes that play very important roles in gluconeogenesis (Hers and De Duve 1950; Chakravarty, Cassuto et al. 2005). During glucose homeostasis in animal, PEPCK1 and G6PC are regulated to response to the blood glucose flux. Insulin is the most important hormone which inhibits PEPCK1 and G6PC expression for the purpose of lowing blood glucose. Insulin-PI3K-Akt-FoxO axis is a major signal pathway which mediates PEPCK1 and G6PC expression. Akt down-regulates PEPCK1 and G6PC expression through phosphorylating FoxO1/3a and in turn inhibiting their transcription activity. Consistent with the roles of FoxO1/3a as the transcription factors for PEPCK1 and G6PC, FoxO proteins binding sites were found on PEPCK1 and G6PC promoter regions (Durham, Suwanichkul et al. 1999; Schmoll, Walker et al. 2000; Altomonte, Richter et al. 2003; Vander Kooi, Streeper et al. 2003; Chakravarty, Cassuto et al. 2005). We cloned 1.5kb promoter region of PEPCK1 and G6PC which contain the FoxO proteins binding sites and studied the promoter activity of those genes in NIH3T3 cells which were transiently transfected with SMP5 wild type or SMP5 DN mutant. Based on luciferase assay, we found FoxO1 or FoxO3a alone can increase the luciferase signal comparing to the empty vector. Co-transfecting SMP5 wild type with FoxO1 or FoxO3a can further enhances the FoxO1/3a transcription activity.

SMP5 DN mutant does not affect FoxO1/3a function based on the readout (Figure 5.1 and Figure 5.2). To confirm whether the enhanced transcription activity of FoxO proteins are directly related to the specific serine site or not, FoxO3a SA SD mutations were generated and co-transfected with SMP5. The data showed the FoxO3a SA mutant luciferase activity cannot be further enhanced by SMP5 since the SMP5 target site has been already mutated to unphosphorylatable status. The transcription activity of FoxO3a SD mutant was not repressed which is out of our prediction (Figure 5.3). The possible explanation is that serine 253 single site phosphorylation is not enough for the FoxO3a nucleus export. It is also reported that acetylation may override the phosphorylation induced nuclear export of FoxO1 (Frescas, Valenti et al. 2005). Furthermore, the SMP5 also cannot significantly affect FoxO3a SD mutant transcription activity. Mutating the serine 253 site can abolish the enhancement of SMP5 on FoxO3a transcription activity suggests serine 253 is the key target of SMP5. The luciferase reporter assay suggests SMP5 can facilitate FoxO1/3a transcription activity on gluconeogenesis related genes and this enhance effect depends on the phosphatase activity of SMP5.

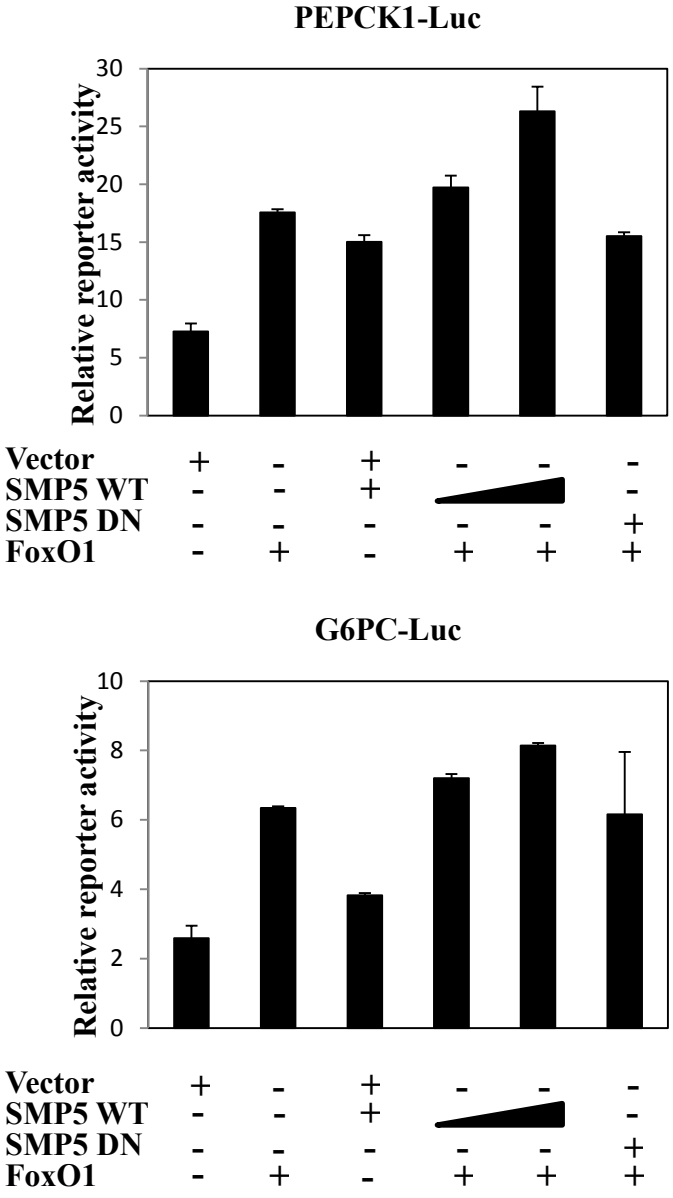


Figure 5.1 **SMP5 enhances FoxO1 transcription activity.** NIH3T3 cells were transfected with PEPCK1 (upper) and G6PC (down) reporter plasmid, FoxO1 and SMP5 wild type and DN mutant. SV-βgal was used as an internal control. 36 hours after transfection, cells were harvested for measurement of luciferase and β-galactosidase activities.

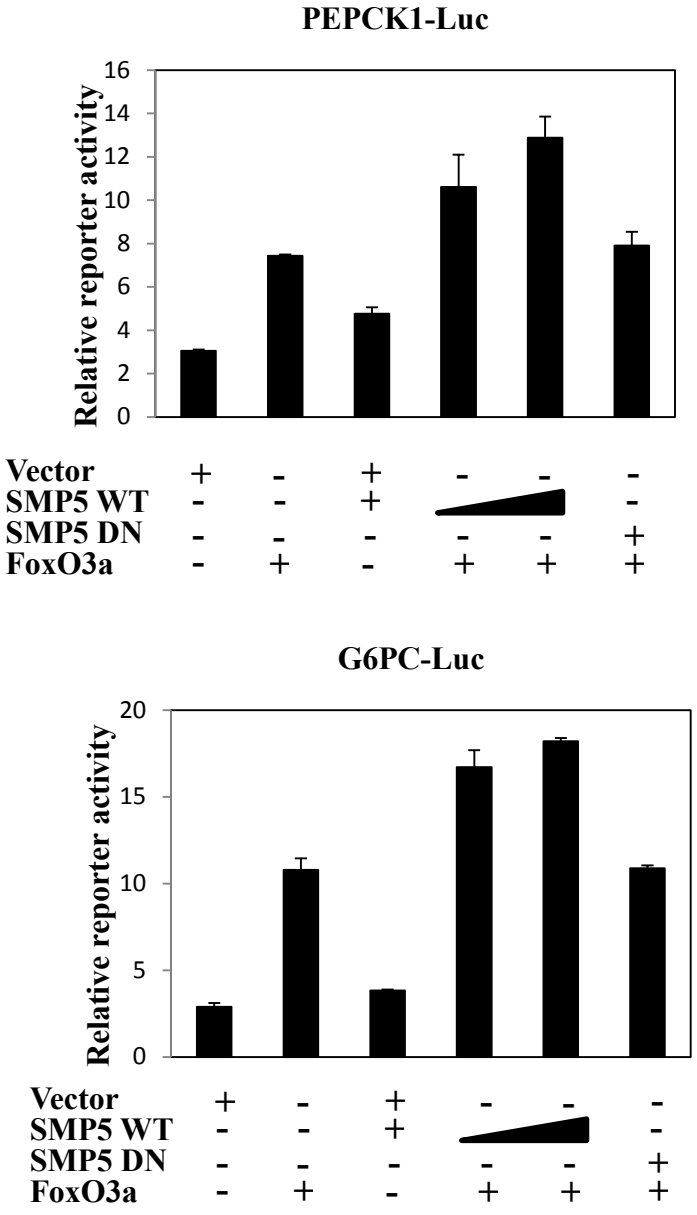


Figure 5.2 **SMP5 enhances FoxO3a transcription activity.** NIH3T3 cells were transfected with PEPCK1 (upper) and G6PC (down) reporter plasmid, FoxO3a and SMP5 wild type and DN mutant. SV-βgal was used as an internal control. 36 hours after transfection, cells were harvested for measurement of luciferase and β-galactosidase activities.

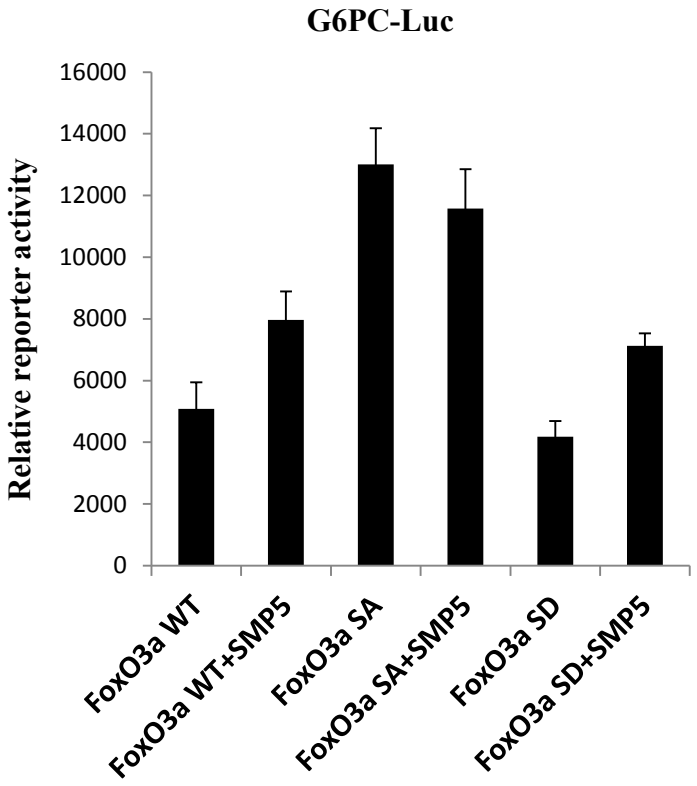


Figure 5.3 **SMP5 enhances FoxO3a transcription activity through dephosphorylating Serine 253.** NIH3T3 cells were transfected with G6PC reporter plasmid, FoxO3a wild type, FoxO3a SA mutant (upper), FoxO3a SD mutant (down) and SMP5 wild type. SV-βgal was used as an internal control. 36 hours after transfection, cells were harvested for measurement of luciferase and β-galactosidase activities.

Given the important roles of PEPCK1 and G6PC in gluconeogenesis process (Lei, Shelly et al. 1993; Shelly, Lei et al. 1993; Chakravarty, Cassuto et al. 2005). The most important consequence of PEPCK1 and G6PC expression level changes is the affected glucose production (Veneziale, Donofrio et al. 1983; Liu, Barrett et al. 1994; Haber, Chin et al. 1995; Mosseri, Waner et al. 2000; Robinson, Dinulescu et al. 2000). To test whether SMP5 can affect gluconeogenesis through regulating FoxO1/3a's transcription activity on PEPCK1 and G6PC, we applied cells based glucose production assay. The liver derived HepG2 cells were used to generate the SMP5 knockdown stable cell line. After incubating with glucose production medium, the cells released glucose was measured and the glucose concentration was normalized with the protein concentration of the whole cell lysate. The data showed that SMP5 knockdown stable cell line produced less glucose compare to control cell line under the same condition (Figure 5.4).

In summary, SMP5 can enhance FoxO1 and FoxO3 transcription activity on PEPCK1 and G6PC, and this ability is related to SMP5's phosphatase activity. Knockdown SMP5 in HepG2 cells can significantly decrease the glucose production of HepG2 cells. The data supports that SMP5 plays important role in gluconeogenesis through FoxO1/3a.

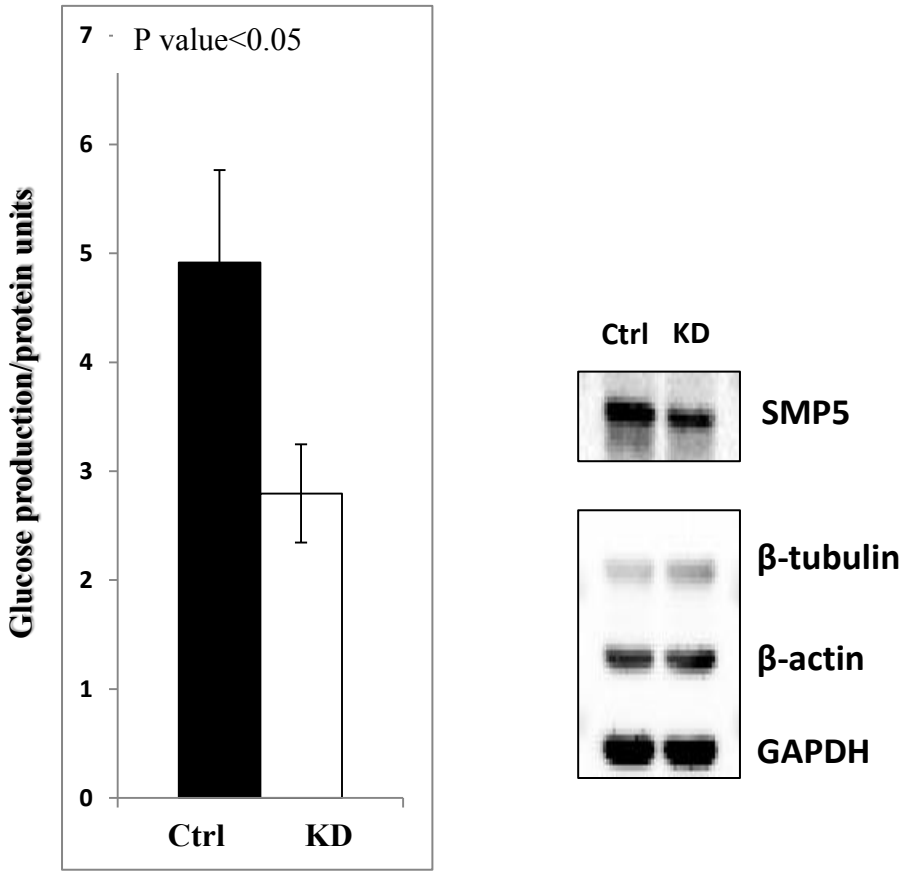


Figure 5.4 **Knocking down SMP5 represses HepG2 glucose production.** HepG2 cells were treated with glucose production medium for 16 hours. 3 hours before measurement, glucagon was added into medium. Medium glucose concentration was measured and normalized by cell lysate protein concentration. (Left panel) SMP5 knock down efficiency was verified by western blotting. (Right panel)

CHAPTER VI

GENERATING AND VALIDATING SMP5 KO MOUSE

INTRODUCTION

Cell based assays showed SMP5 can dephosphorylate FoxO1/3a and affect gluconeogenesis related genes expression. The SMP5 function in animal is needed to be further answered. Manipulating endogenous SMP5 level in mouse gave us an opportunity to understand the role of SMP5 *in vivo*. For this purpose, a SMP5 knockout mouse was generated by our laboratory by using plasmid purchased from KOMP with gene trap strategy (Gossler, Joyner et al. 1989). The deletion of SMP5 gene was validated with qPCR and western blotting.

MATERIALS AND METHODS

GENE TRAP MOUSE

A lacZ gene contained cassette was inserted into the intron 2 of SMP5. The transcription terminator inside the insertion cassette can block the transcription of SMP5 mRNA from genome after intron 2 (Figure 6.1).

MEF CELLS ISOLATION

Mouse embryonic fibroblast (MEF) cells were isolated from wild type and SMP5 KO mouse embryos at developmental stage E12.5~E13.5. All protocols are approved by the Institutional Animal Care and Use Committee (IACUC) to maintain and use of laboratory animals. All equipment which contact with embryos and cells were

autoclaved and the solutions were filtered with 0.2μm membrane. MEF cells were isolated as following steps:

1. Pregnant mice were sacrificed 12.5 to 13.5 days after plug was observed and the fetuses were dissected out.
2. The tails of fetus were kept for genotyping. The head, limbs and major organs including heart, lung, liver and kidney were removed from embryo. The trunk was washed with PBS to remove the remaining blood.
3. Trunk was transferred to 10mL tubes and cut into small pieces with forceps.
4. 0.25% trypsin was added into tubes and incubating the cut trunk for 15 minutes at 37°C.
5. 10% FBS DMEM medium was added into tube and pipet vigorously to break down the tissue into cells suspension.
6. Precipitating the cell suspensions by gravity for 1 minute to let remaining clumps sinking to the tube bottom.
7. The supernatant was transfer to new tubes and spun down with 200g for 2 minutes, the cell pellet was washed with fresh medium and spun down again.
8. Cells were re-suspended with 10% FBS DMEM medium and seeded into 10cm dish.
9. After overnight culture, medium was changed and all suspending cells were removed with PBS washing. The remaining attached cells were MEF cells.

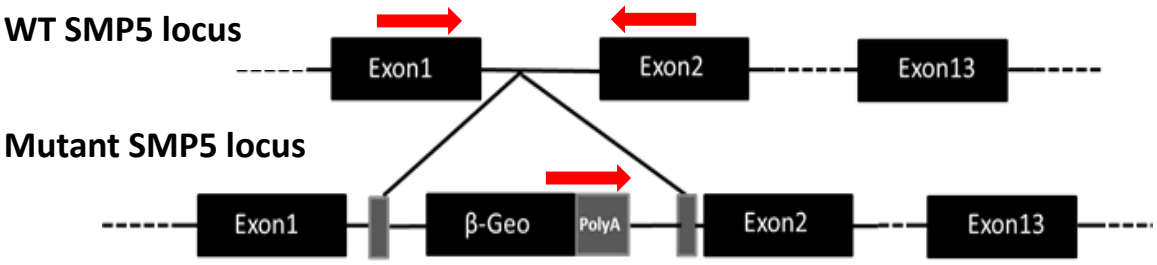


Figure 6.1 **Strategy of generation SMP5 gene trap mutant mice in C57BL/6 strain.** 13 exons were included in SMP5 transcripts and a prepared cassette was inserted into intro 2. With the stop code in cassette, transcription of SMP5 will be stopped immediately after exon 1 and IRES in cassette can help to transcript the β-geo gene which is fused to the incomplete SMP5 transcripts.(Red arrows present the primers for genotyping)

GENOTYPING

2~3 mm mouse tail was cut for genome DNA extraction. The tail was lysed with 100 µl 50mM NaOH at 100°C for 30 minutes. The lysate was neutralized with 9 µl 1M Tris.HCL pH6.5. 1.5 µl neutralized lysate was used as the DNA template for genotyping with genotype primers (Table 2.1).

WESTERN BLOTTING

MEF cells were cultured in 6 wells plate to reach 70% confluence. Harvest the cells with 2XSDS western loading buffer and boil the lysate for 10 minutes. Sample was subjected to western blotting to verify the SMP5 protein level.

RESULTS AND DISCUSSION

To study the role of SMP5 in mouse, gene trap strategy was used to generate the whole body SMP5 knockout mouse. SMP5 knockout mouse was gotten successfully and MEF cells were isolated to validate the knockout efficiency. First of all, the genotyping primers were test and the PCR product bands were separated and detect by 2.0% Agarose gel electrophoresis. In figure 6.2, wild type, heterozygous and homozygous were represented by different band patterns. The wild type mouse only has one band with small size, the homozygous has one band with large size and the heterozygous has both small size band for wild type allele and big size band for knockout allele. Secondary, qPCR was applied to verify the SMP5 mRNA level in MEF cells. The primers were designed to target sequence after exon 2 which should be deleted in homozygous due to the transcription terminator on LacZ cassette insertion in intron 2. We found there is only trace amount of SMP5 mRNA can be detected in homozygous

comparing to wild type littermate control. Finally, we checked the protein level of SMP5 in MEF cells, the western data showed that the SMP5 protein level is dramatically decreased in homozygous comparing to control but there is still detectable SMP5 protein in homozygous MEF cells. Based on the qPCR and western blotting data, we confirmed that the SMP5 level is significantly decreased in homozygous. The remaining trace amount of SMP5 protein which is found in homozygous MEF cells may due to the expression leaky of SMP5. Considering the dramatically decrease in SMP5 protein level in homozygous, the mouse line is suitable for further studying the physiological role of SMP5 in animal.

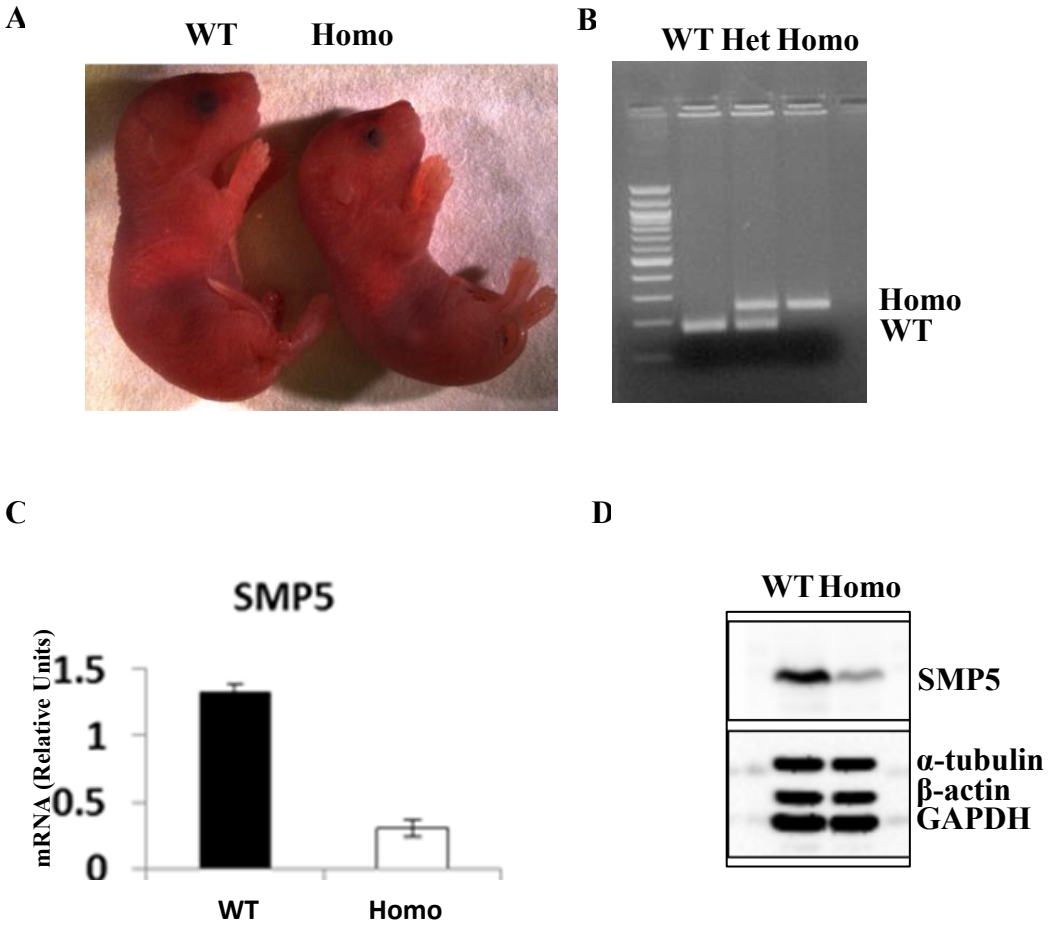


Figure 6.2 **Generation of SMP5 knockout mice.** SMP5 knockout mouse was generated with LacZ insertion. (A) Targeting vector was inserted into SMP5 intron 2 and the insertion was verified by PCR. (B) Deletion of SMP5 gene was verified by qPCR (C) and western blotting (D) in MEF cells.

CHAPTER VII

SMP5 KNOCKOUT MOUSE HAS NEONATAL LETHAL PHENOTYPE WITH DEFECTED GLUCONEOGENESIS

INTRODUCTION

SMP5 knockout mouse was generated to explore the SMP5 function in animal. The SMP5 expression pattern was profiled by using In Situ hybridization. Since SMP5 was found to affect glucose production through regulate FoxO1/3a phosphorylation status, we explored the phenotype of the SMP5 knockout mouse, especially focus on glucose homeostasis of the homozygous.

MATERIALS AND METHODS

IN SITU HYBRIDIZATION

To reveal the expression pattern of SMP5 in mouse whole body, SMP5 cDNA was re-cloned into pBluescriptKS and pBluescriptSK as the template for In situ hybridization RNA probes synthesis. 20μg pBluscriptKS SMP5 and pBluscriptSK SMP5 were digested with BamHI at 37°C. The linearized DNAs were purified with phenol-choloroform. After concentration measuring, 3.6μg DNA templates were used to generate Digoxigenin labeled probes by using the Digoxigenin labeling kit (Roche Cat#11277073910) and T3 or T7 reverse transcriptase. The DNA templates were removed by DNaseI digesting and RNA probes were precipitated with 75% pre-cooled EtOH containing 0.4M $LiCl_2$ at -80°C. The probe pellets were washed with 75% EtOH

and dissolved with DEPC water. The dissolved probes were treated with hydrolysis buffer (0.06M Na_2CO_3, 0.04M $NaHCO_3$) at 60°C for 20 minutes to get smaller size probes (around 300 bps) to enhance the hybridization efficiency. After neutralized the reaction mixture with neutralization buffer (0.2M $NaC_2H_3O_2$, 1% acetic acid) the probes were precipitated and re-suspended with DEPC water. Followed with concentration measurement, probes were aliquoted into individual tubes for further using.

The pregnant mouse was sacrificed at 16 days after plug was observed. The embryos were fixed with 4%PFA and followed with standard protocol for dehydration and wax embedding. 5μm sagittal sections were cut for In Situ hybridization.

For *In Situ* hybridization, the sections were treated with standard de-waxing and re-hydration protocol (all staffs are RNAase free). The PBS re-hydrated slides were treated with proteinase K (Proteinase K 30μg/ml, 0.05M Tris-HCl pH8.0, 50μM EDTA pH8.0) for 10 minutes at room temperature. The slides were post-fixed with 4%PFA for 20 minutes at room temperature and washed twice with PBS and then washed twice with 2X SSC buffer (150mM NaCl, 15mM trisodium citrate, pH 7.0). Then, the slides were pre-hybridized with hybridization buffer (50% Formamide, 5XSSC, pH4.5~5, 1% SDS, 50μg/ml yeast tRNA, 50μg/ml heparin) without probes for 2 hours at 55°C. Sense and antisense SMP5 probes were diluted in hybridization buffer to final concentration 400ng/ml. The probes were heated for 95°C for 2 minutes on heat block and chilled on ice immediately to dehybridize the RNA probes. 50μl hybridization solution was applied to the section at 50°C overnight with humidity chamber. After probes hybridization, slides were washed with SSC washing buffer (2 X SSC, 50% formamide) for 2 X 30

minutes at 55°C. Then, slides were washed with 1X MABT (100 mM maleic acid, 150mM NaCl, 0.1% Tween20, pH 7.5) for 2 X 30 minutes at room temperature. The washed sections were blocked with blocking buffer (2% block reagent: Roche cat# 11096176001 in 1X MABT with 20% heat inactivated sheep serum) for 2 hours at room temperature and followed with 1:250 dilution of anti-DIG alkaline phosphatase antibody (Roche cat# 11093274910) in a humidified chamber at 4°C overnight. The slides were washed with 2μM levamisole contained MABT for 5 X 30 minutes at room temperature and followed with NTMT(100mM NaCl, 100mM Tris-HCl pH9.5, 50mM $MgCl_2$, 1% Tween 20) washing for 2 X 5 minutes at room temperature. After washing, the sections were incubated with BM purple substrate (Roche Cat#11442074001) in dark humidity chamber for signal development. The sections were washed with PBS and post-fixed with 4%PFA for 20 minutes to stabilized staining. Finally, the slides were washed with PBS and mounted with aqueous mounting solution.

BLOOD GLUCOSE MEASURMENT

Blood was collected from natural delivered P0 pups or E18.5 embryos from C-section. Blood glucose concentration was measured with Contour® Blood Glucose Meter.

GLUCOSE AND PYRUVATE INJECTION RESCUE

E18.5 embryos were dissected out with C-section and injected with 20μl glucose (10% glucose and 0.9% sodium chloride in dd H_2O) or 20μl pyruvate (10% pyruvate and 0.9% sodium chloride in dd H_2O) every 6~8 hours. Mouse status was checked every one hour.

RNA AND PROTEIN EXTRACTION

Liver and pancreas were dissected out from sacrificed mouse. After rising with PBS, liver was separated to two parts for RNA and protein respectively. For protein sample, the liver was homogenized in cold RIPA buffer with protease inhibitor and centrifuged with 12,000 rpm for 10 minutes at 4°C. The supernatant was kept and the concentration was measured with Bio-Rad protein assay kit (Cat#500-0001). For RNA sample, the liver and pancreas were homogenized in 1ml cold Trizol solution and kept at room temperature for 5 minutes to complete dissociate nucleoprotein complexes. 200μl chloroform was added into tube and mixed well with shaking tube vigorously by hand for 15 seconds. The mixture was incubated at room temperature for 2 to 3 minutes. Then the sample was centrifuged with 12,000g for 15 minutes at 4°C. Supernatant was collected and 0.5ml isopropyl alcohol was added to precipitate total RNA. After spin down, the pellet was washed with 70% EtOH and re-suspended with DEPC water. The RNA concentration was measured with spectrometry.

QPCR

1μg RNA was used for template and multiple genes were checked by using primers in the list. (Table 2.1) 18S was used as the internal control.

WESTERN BLOTTING

Protein samples were subject to different concentration SDS PAGE for different sizes protein molecules. 13.5% SDS PAGE was used for LC-3, 10% SDS PAGE was used for other proteins.

LIVER FRACTIONATION

After 12 hours fasting, mouse was sacrificed and liver was dissected out. Liver was transferred into 1ml VWR® Dounce homogenizer pre-chilled on ice with 1ml A buffer containing protease cocktail (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF, Na3VO4). Grinding the liver gently with 4 times push. Lysate was spin down with 1,200g for 10 minutes at 4°C. The pellet was washed with 1ml A buffer and spin down with 1,200g for 5 minutes at 4°C. Pellet was lysed with 200µl buffer B containing protease cocktail (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF, Na3VO4) for the nucleus fractionation.

IMMUNOHISTONCHEMISTRY

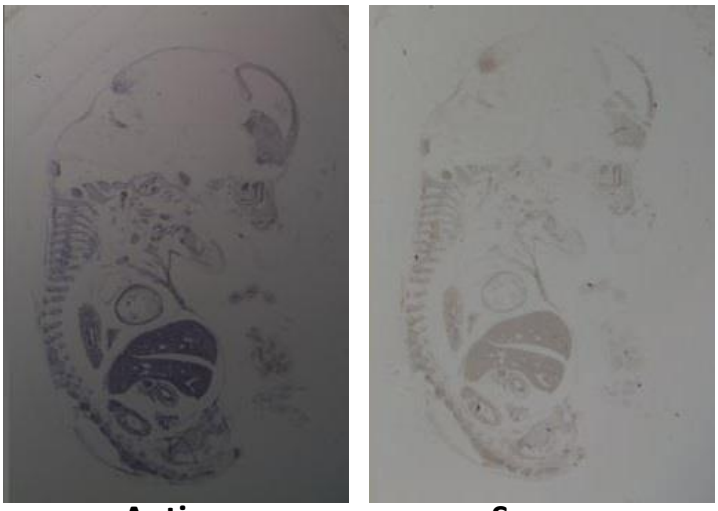
Liver sample was fixed with 4%PFA. The fixed sample was dehydrated and embedded with standard protocol. 5µm section was cut for immunohistochemistry.

PSA STAINING

5µm sections were treated with standard de-waxing and re-hydration process. After re-hydration, sections were oxidized with 0.5% periodic acid solution (Sigma Cat#7875) for 5 minutes and rinsed in distilled water. Schiff reagent (Sigma Cat#3952016) was applied to sections for 20 minutes and removed with tap water washing for 5 minutes. Slides were counterstained with hematoxylin for 1 minute and washed with tap water for 5 minutes. Finally, slides were dehydrated and mounted with synthetic mounting medium.

RESULTS AND DISCUSSION

To profile the expression pattern of SMP5, *In situ* hybridization approach was used by using probe synthesized from SMP5 cDNA template. As shown in Figure 7.1, positive signal can be detected by using anti-sense probe but cannot be detected with sense probe which suggests the bind between probe and target mRNA is specifically. Based on the signal distribution pattern, we found that SMP5 is expressed in most of the major organs and tissues including brain, heart, lung, liver, kidney and intestine. To further investigate SMP5 function *in vivo*, the SMP5 knockout mouse was generated from a gene trap C57BL/6 ES cell line. Interestingly, the SMP5 knockout mouse dies within 24 hours after birth. Due to the neonatal lethal phenomenal, mice heterozygous for the SMP5 deletion were used to keep the line. The day 1 progeny of heterozygous breeding was analyzed by PCR (Figure 6.2B). At day 1, besides the slightly small body size of the homozygous the progenies were phenotypically similar to their heterozygous and wild type littermates and the progenies displayed the expected Mendelian ratio of mice heterozygous and homozygous for the SMP5 deletion. The Mendelian ratio at P0 suggests the SMP5 knockout can survive until deliver. Mouse neonatal lethality can be caused by numerous defects. Lung, cardiovascular, neuromuscular and skeletal defects can cause respiration problems and the neonate has these defects will undergo asphyxiation and die soon after birth. Craniofacial defects and parental behavior also play important roles in mouse neonatal lethal phenomenal by which the neonate cannot get the milk supply successfully. Finally, both the skin defect and renal failure can cause body fluid homeostasis problem and glucose homeostasis related hypoglycemia can

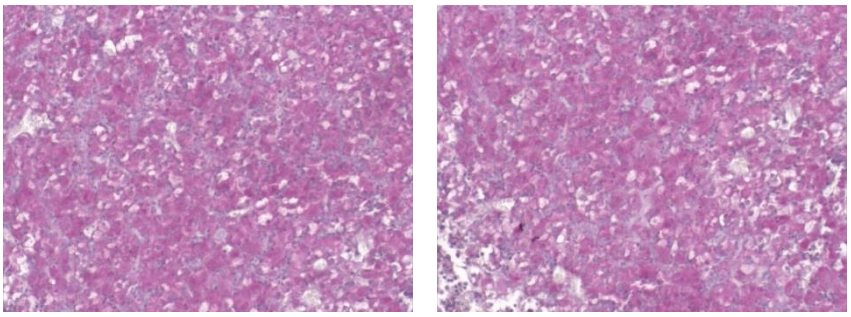


C

Figure 7.1 **SMP5 expresses in major organs of wild type mouse.** E16.5 C57BL/6 wild type mouse embryo sagittal section was hybridized with antisense (A) and sense (B) SMP5 probes. (C).Dot blotting for antisense and sense probes concentration verification.

cause the neonatal lethality due to the physiological homeostasis defaults (Turgeon and Meloche 2009). Since we found that SMP5 can affect glucose product in cells through regulating FoxO1/3a, we mainly focused on checking the blood glucose of the neonates. The blood glucose measurement showed that immediately after birth the readouts are comparable between wild type and homozygous. It is reasonable since the blood glucose is mainly maintained by mother before deliver and the neonates can keep the blood glucose level at a stable level for a while with the maternal derived glucose. The trans-placental nutrient and energy supply are immediately terminated at birth, neonates have to deal with the cessation of nutrient until supply can be restored with milk (Kuma, Hatano et al. 2004). During the starvation period, glycogenolysis and gluconeogenesis are important to support the glucose homeostasis (Darlington 1999). After caesarean delivery, the pups were sacrificed and the livers were subjected to frozen section. PSA staining was applied to the frozen sections to check the hepatic glycogen storage of the new born pups. The staining result did not show obvious difference between wild type and SMP5 knockout mice samples (Figure 7.2). To mimic the nutrient cessation, the neonates from caesarean delivery were starved for 12 hours and the blood glucose level was measured by using blood glucose meter. Consistent with the role of SMP5 in cell based glucose production, the SMP5 knockout has significantly decreased blood glucose comparing to the littermates control (either wild type or heterozygous) (Figure 7.3). To further confirm whether the lethal phenotype of SMP5 knockout is due to the decreased blood glucose, glucose injection rescue was set up and the data showed that the survive

time is increased in SMP5 knockout pups with glucose injection comparing to the knockout pups with saline injection only (Figure 7.4). This result suggests exogenous glucose input can at least partially rescue the lethal phenotype of SMP5 knockout moues.



WT **Homo**

Figure 7.2 **Glycogen storage is not affected in SMP5 Knockout mouse liver.** P0 mouse liver was fixed and stained with PAS. Pink color represents the glycogen.

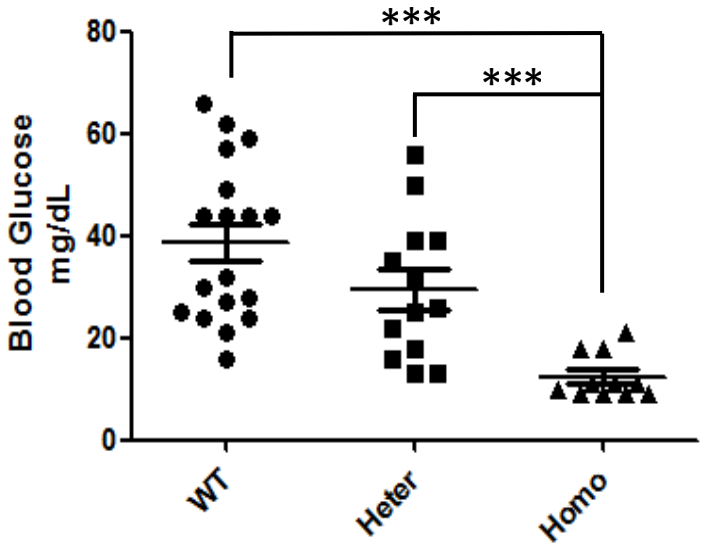


Figure 7.3 **SMP5 knockout mouse blood glucose is significant lower than control.** After caesarean section, the mouse embryos were starved for 12 hours at 30°C in a humidity chamber. The blood samples were collected from embryo tail and measured with blood glucose meter. (***: P value<0.001)

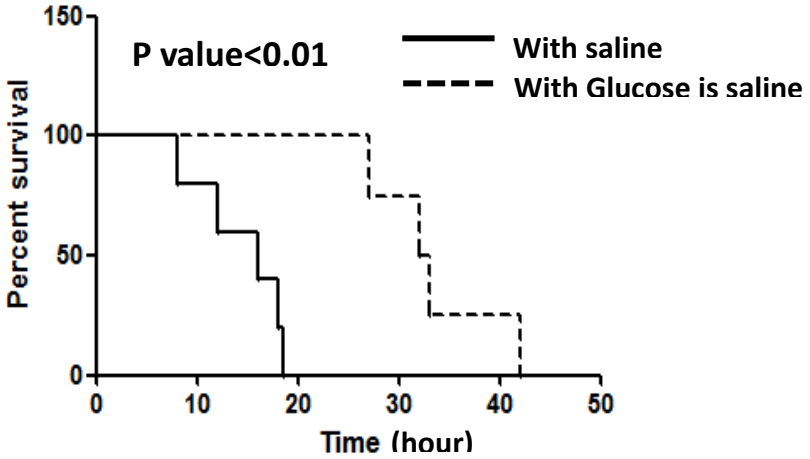


Figure 7.4 **Glucose injection can elongate SMP5 knockout survive time.** After caesarean section, the mouse embryos were injected with glucose every 6~8 hours and observed every hour at 30°C in a humidity chamber.

Besides liver, SMP5 expression also presents in other major organs such as brain, heart and lung. The multiple organs expression suggests SMP5 not only play a role in liver for glucose homeostasis, it also may have other important roles in different organs. The partial but not fully rescue with glucose injection may due to the potential complexity of SMP5 functions. Pancreas secreted hormones including insulin and glucagon are important molecules which regulate blood glucose level. To investigate whether the dropping blood glucose in SMP5 knockout mouse is due to the abnormal insulin or glucagon secretion, pancreas insulin and glucagon mRNA level were verified with qPCR and no significant difference was found in homozygous mouse samples (Figure 7.7). Multiple genes which are related to hepatic glucose production were checked by using qPCR. We found the glycogenolysis and glycogenesis related genes are not changed in SMP5 homozygous liver, but consistent to the cell based assay expression of PEPCK1 and G6PC which are important for gluconeogenesis are significantly decreased (Figure 7.6). The pyruvate injection cannot elongate the survive time of SMP5 knockout mice also support the hypothesis that the blood glucose decreasing is due to insufficient PEPCK1 in SMP5 knockout mouse liver since pyruvate is the substrate for glucose synthesis (Figure 7.5). Autophagy in SMP5 knockout mouse liver was checked to further exclude the possibility of substrates for gluconeogenesis since autophagy is important for the fasting neonate to generate substrates for gluconeogenesis such as glucogenic amino acids (Kuma, Hatano et al. 2004). Western blotting of autophagy marker LC-3 showed that autophagy is normal in SMP5 knockout

mouse which suggests the substrates supply of gluconeogenesis is not affected in SMP5 knockout mouse (Figure 7.8).

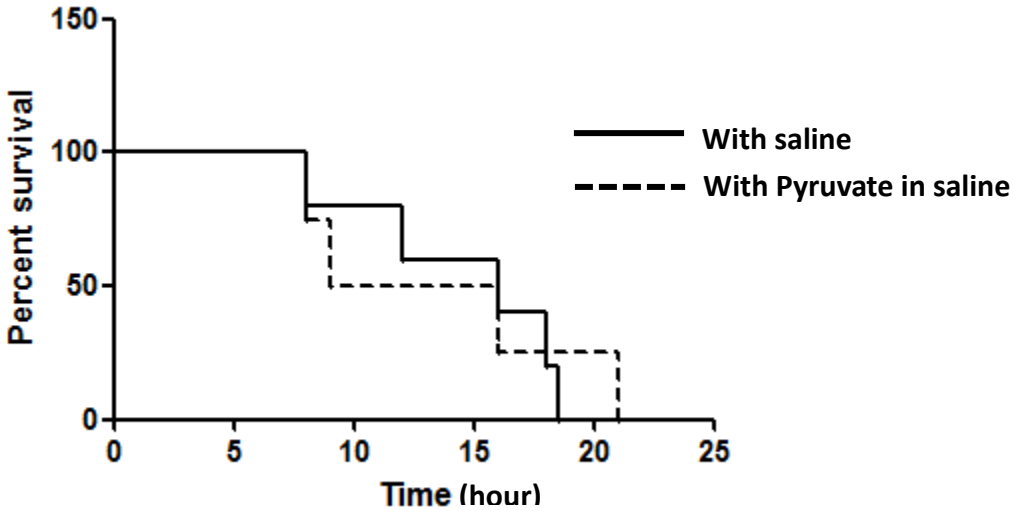


Figure 7.5 **Pyruvate injection cannot elongate SMP5 knockout survive time.** After caesarean section, the mouse embryos were injected with pyruvate every 6~8 hours and observed every hour at 30°C in a humidity chamber.

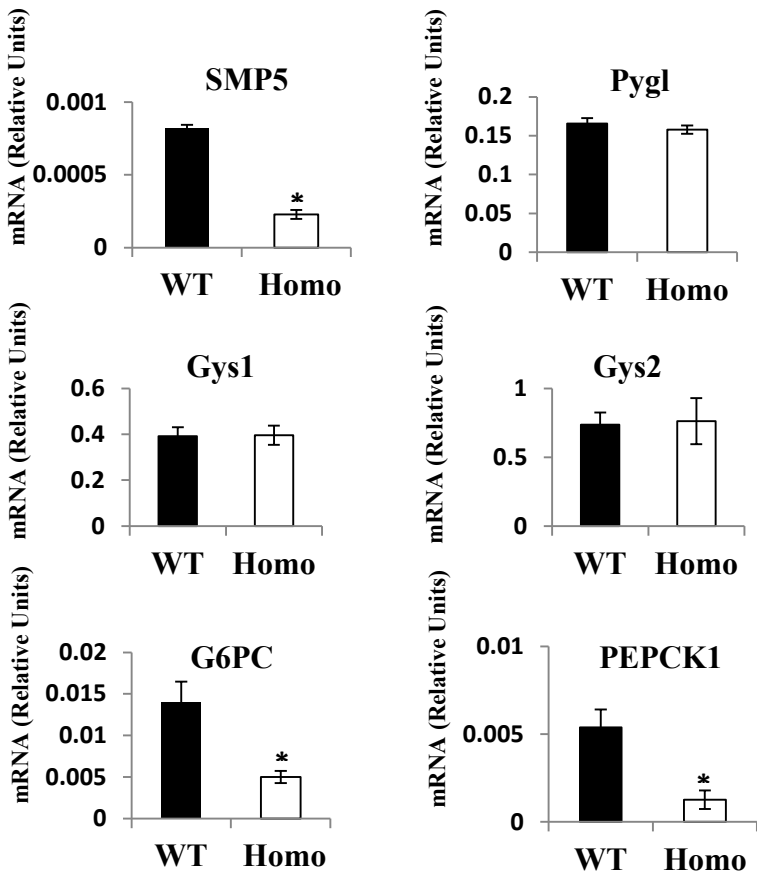


Figure 7.6 **PEPCK1 and G6PC expression are decreased in SMP5 knockout mouse liver.** Glucose metabolism related genes were analyzed with quantitative PCR. Pygl, Gys1 and Gys2 expression level are comparable for wild type and SMP5 knockout. PEPCK1 and G6PC expression are decreased in SMP5 knockout mouse liver.

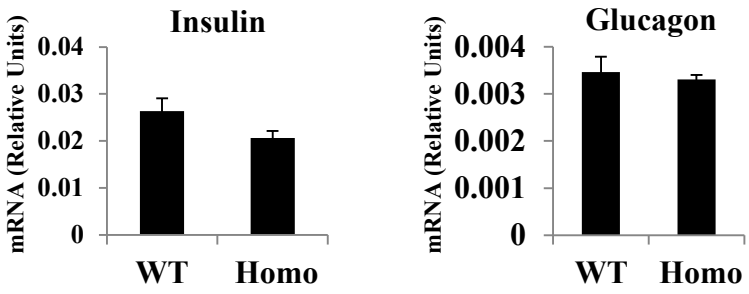


Figure 7.7 **Hormones mRNAs level is not affected in SMP5 knockout mouse pancreas.** Insulin and Glucagon mRNA level was measured by qPCR and no significant difference was found in SMP5 knockout mouse pancreas.

The expressions of major transcription activators and co-factors were also be tested with qPCR, the data showed that like other genes the expression level of FoxO1/3a does not change in SMP5 knockout mouse (Figure 7.9). Because SMP5 was shown to be a phosphatase of FoxO1/3a in cell based system, serine256/253 of FoxO1/3a respectively were checked in SMP5 knockout mice and their littermates' livers. Not surprisingly, phosphorylation of serine256/253 is increased in knockout mice (Figure 7.10). Akt is an important FoxO1/3a kinase which can phosphorylate FoxO1/3a at serine265/253 respectively and regulate FoxO1/3a transcription activity (Paradis and Ruvkun 1998; Brunet, Bonni et al. 1999; Rena, Guo et al. 1999). Total Akt and Akt activated form (pS473 Akt) were also tested, western blotting showed the total Akt does not change and activate form is slightly decreased in SMP5 knockout liver which means the phosphorylation increasing of FoxO1/3a is not due to the increase of kinase activity (Figure 7.10). Increasing phosphorylation of FoxO1/3a can enhacne their nucleus export, liver FoxO3a subcellular distribution was checked with tissue fractionation and immunohistochemistry. Both fractionation data and immunohistochemistry resulte showed the nucleus localized FoxO3a is decreased in SMP5 knockout mouse liver (Figure 7.11 and Figure 7.12).

In summary, SMP5 knockout mice die within 24 hours after birth with severe hypoglycemia. The hypoglycemia is due to defect in gluconeogenesis related genes, PEPCK1 and G6PC, expression. The decreasing of PEPCK1 and G6PC is caused by repressiong FoxO1/3a transcription activity through decreasing FoxO1/3a dephosphorylation.

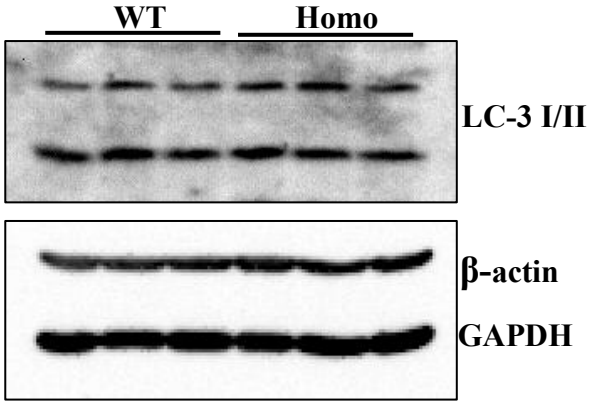


Figure 7.8 **Autophagy is not affected in SMP5 knockout mouse liver.** Paired littermates liver lysates were harvested from 12 hours fasted neonates and subjected to western blotting using LC-3, β-actin and GAPDH antibodies as indicated.

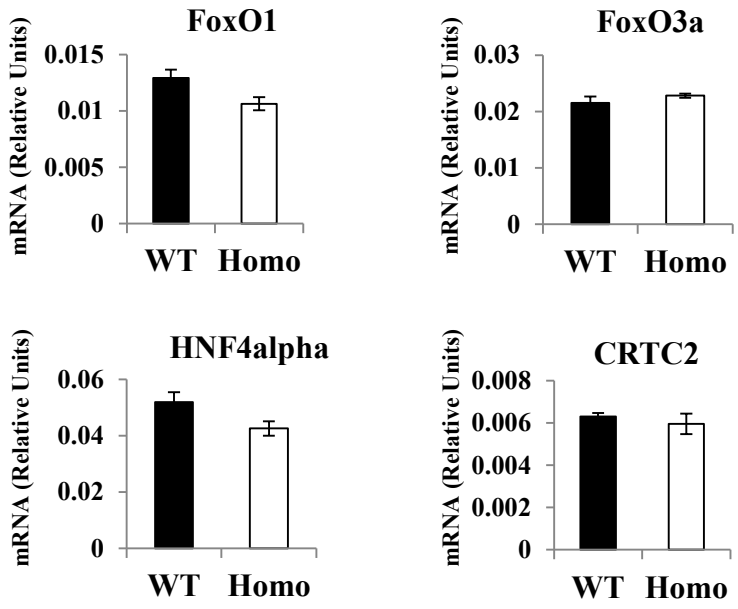


Figure 7.9 **Important transcription factors and co-activators are not affected in SMP5 knockout mouse liver.** RNA samples were isolated from neonates after 12 hours starvation and genes expression were measured with qPCR.

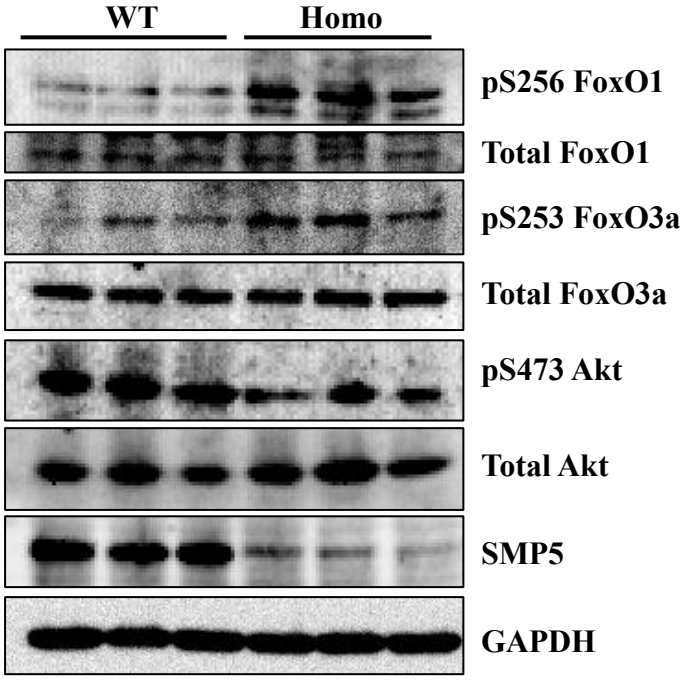


Figure 7.10 **Phosphorylation of FoxO1/3a is increased in SMP5 knockout mouse liver and the increase is not due to increasing Akt activity.** Livers from neonates after 12 hours starvation were lysed and subjected to western blotting using phospho-FoxO1, FoxO1, phospho-FoxO3a, FoxO3a, phospho-Akt, Akt, SMP5 and GAPDH as indicated.

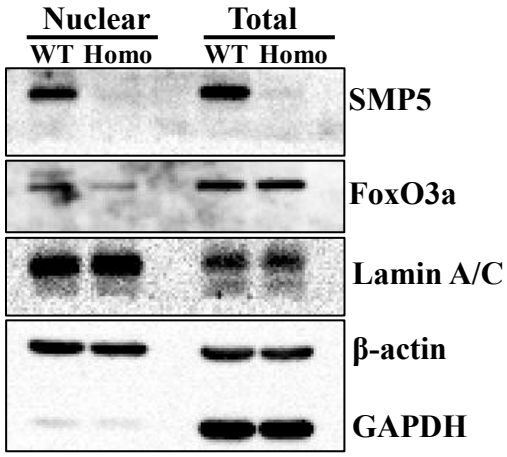


Figure 7.11 **Nucleus localized FoxO3a is decreased in SMP5 knockout mouse liver.** Fractionation liver samples from neonates after 12 hours starvation were subjected to western blotting using SMP5, FoxO3a, Lamin A/C, β-actin and GAPDH as indicated.

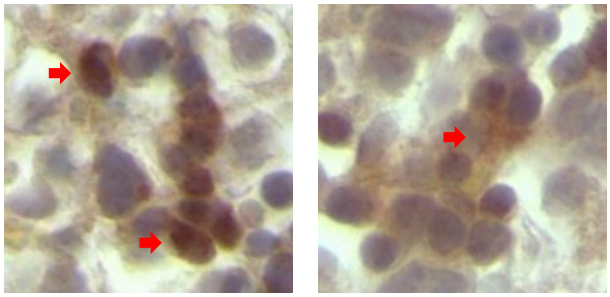


Figure 7.12 **Nucleus localized FoxO3a is decreased in SMP5 knockout mouse liver.** Sections from neonate liver after 12 hours starvation were stained with FoxO3a antibody and counter stained with hematoxylin. Red arrows indicate nucleus and cytoplasm localized FoxO3a.

CHAPTER VIII

CONCLUSION AND FUTURE DIRECTION

INTRODUCTION

Gluconeogenesis is a physiological process through which the organ can *de novo* synthesize glucose through non-carbohydrate carbon substrates including pyruvate, lactate, glucogenic amino acids and fatty acid. It is the major compensation mechanism for stabilizing blood glucose level after the body is running out of glycogen storage during prolonged starvation. PEPCK1 and G6PC are two key rate-limiting enzymes which control the speed and yield of gluconeogenesis. Regarding the important roles of PEPCK1 and G6PC, the expressions of PEPCK1 and G6PC are tightly regulated *in vivo* in order to keep the stable blood glucose level. Multiple transcription factors and co-factors are reported to regulate the expression of PEPCK1 and G6PC. FoxO1 and FoxO3a are two important factors of these transcription factors which regulate PEPCK1 and G6PC genes expression. There are Ser/Thr sites distribute on FoxO proteins which can be phosphorylated by different Ser/Thr kinase. After phosphorylating by Akt/PKB, FoxO proteins will be exported from nucleus to cytoplasm and then the transcription activity of FoxO is repressed (Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999). Controversy to the repression effect of Akt, there are phosphatases can antagonize the inhibition of Akt through dephosphorylating the sites which are phosphorylated by Akt. Phosphatases PP2A and MKP3 (DUSP6) can dephosphorylate FoxO1 and facilitate its nucleus sustaining (Yan, Lavin et al. 2008; Wu,

Jiao et al. 2010). MKP3 was reported to be a cytosolic phosphatase for FoxO1 and PP2A was shown to be a phosphatase of FoxO1 with role in cell apoptosis (Xu, Yang et al. 2005; Yan, Lavin et al. 2008). Since the FoxO1/3a kinas Akt can be activated in nucleus (Wang and Brattain 2006; Villarejo-Balcells, Guichard et al. 2011), in principle, it is possible that there is nucleus located phosphatase exist which regulate FoxO1/3a in glucose homeostasis.

SUMMARY

In this study, we identified SMP5 (a member of the small C-terminal domain phosphatase) as a novel FoxO1/3a phosphatase. Interestingly, SMP5 protein level is regulated during fasting/feeding cycle in wild type mouse which suggests its role in metabolism homeostasis (Figure 2.1). Overexpression of SMP5 leads to reduction of the endogenous phospho-FoxO1/3a and this effect depends on the phosphatase activity (Figure 2.4). Knocking down SMP5 can increase the endogenous phospho-FoxO1/3a (Figure 2.5). Furthermore, purified recombinant SMP5 can directly dephosphorylate phospho-FoxO1/3a *in vitro* (Figure 2.6). These results suggest that SMP5 is a phosphatase for dephosphorylation of FoxO1/3a in the PI3K-Akt axis.

DISCUSSION

Unlike MKP3 which is also a FoxO1 phosphatase located in cytoplasm (Wu, Jiao et al. 2010), SMP5 is a nucleus localized protein (Figure 2.2 and 2.3). Overexpressing SMP5 can enhance FoxO1/3a nucleus sustaining through dephosphorylating FoxO1/3a and in turn disrupting interaction between FoxO1/3 and 14-3-3ζ (Figure 4.4). Given the

role of FoxO1/3a in PEPCK1 and G6PC transcription regulation, the glucose production is repressed in SMP5 knock down HepG2 cells (Figure 5.4). Consistent with the role of SMP5 in cell, phospho-FoxO1/3a is significantly increased and in SMP5 knockout mouse liver and the gluconeogenesis related genes expression is decreased as a consequence (Figure 7.6 and 7.10). The animal based experiment data indicates the role of SMP5 in hepatic glucose production through regulating FoxO1/3a activity. Gluconeogenesis is important for blood glucose homeostasis especially for the animal or human undergoing prolonged starvation. Besides the role of SMP5 in perinatal starvation related glucose homeostasis, it is worthwhile to further investigate the function of SMP5 in adult mouse or human glucose metabolism. The creation of SMP5 conditional knockout mouse is a suitable approach to study its role in glucose homeostasis in adult mouse since the whole body SMP5 knockout homozygous has neonatal lethal phenotype.

FUTURE DIRECTION

Interestingly, the SMP5 knockout mouse died within 24 hours after birth and glucose injection can only extend the survive time for about 20 hours more. The partially rescue with glucose injection suggests glucose metabolism defect may not be the only problem in SMP5 knockout mouse. Regarding the broad expression pattern of SMP5, it is possible that SMP5 has other roles in different organs since as the substrates of SMP5, FoxO1/3a, also express in multiple tissues and organs (Hosaka, Biggs et al. 2004). Additionally, besides FoxO proteins SMP5 may have other substrates as a phosphatase,

since based on the kinases to phosphatases ratio a protein phosphatase must have 60 to 70 substrates *in vivo* as average.

SMP5 knockout mouse was further characterized at different stage of embryo development. Besides liver, the other major tissues and organs were checked including brain, heart, lung and kidney. Ventricle septum defect (VSD) was found in SMP5 knockout mice heart and the penetration is around 70% (Figure 8.1 and Figure 8.2). The involvement of FoxO proteins in cardiovascular development has been found by different laboratories. FoxO1 knockout mouse dies before birth by day E10.5 to E11 with grossly vascular and cardiac development defects (Furuyama, Kitayama et al. 2004). Interestingly, the cardiac specific ectopic expressing FoxO1 can cause the embryonic lethal at E10.5 with heart failure which due to the impaired cardiomyocyte proliferation and reduced myocardium thickness (Evans-Anderson, Alfieri et al. 2008). The knockout and overexpression FoxO1 induced embryonic heart development defects suggest the sophistically regulation of FoxO1 activity is required for the heart development. FoxO3a involves the endothelial cells migration and sprouting during development through repression BMPER (BMP-binding endothelial cell precursor derived regulator) expression (Moser, Binder et al. 2003; Heinke, Wehofsits et al. 2008). Given the role of SMP5 as a phosphatase of FoxO1/3a, the function of SMP5 in cardiovascular development is need to be further studied.

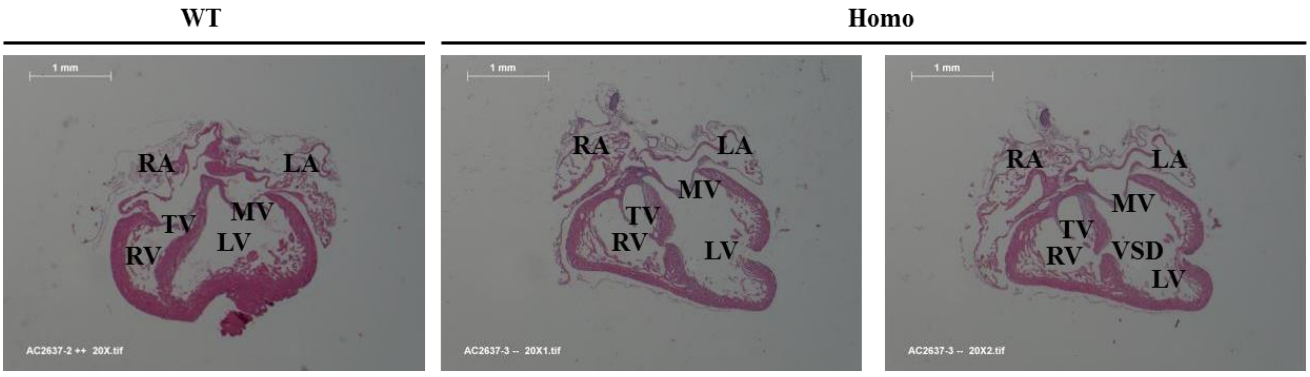


Figure 8.1 **VSD is found in SMP5 knockout mouse heart I.** C-section delivered mice were perfused with PBS and fixed with PFA through Venae umbilicalis. Wax embedded hearts were subjected to cross section and HE staining. (RA: right atrium, LA: left atrium, TV: tricuspid valve, MV: mitral valve, RV: right ventricle, LV: left ventricle, VSD: ventricle septum defect)

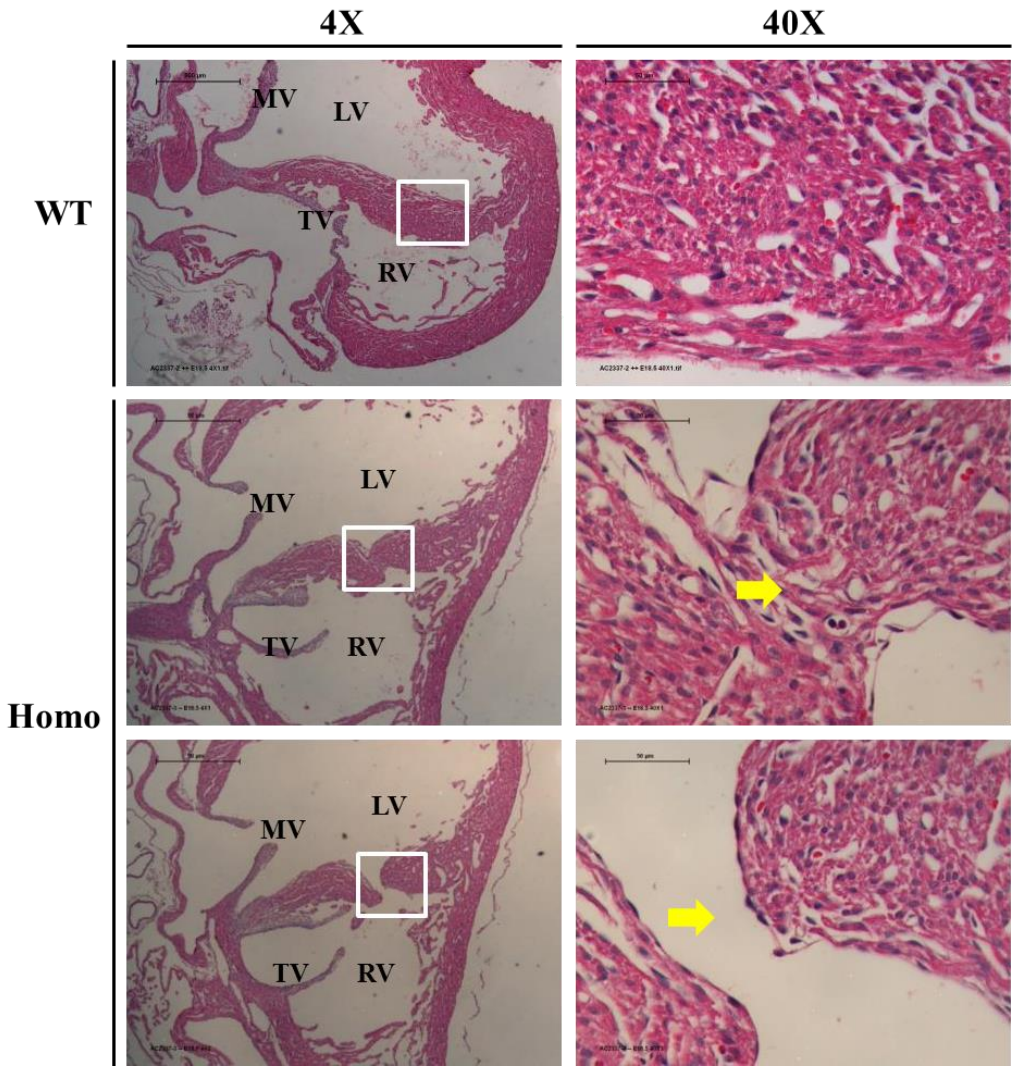


Figure 8.2 **VSD is found in SMP5 knockout mouse heart II.** Amplification of Figure 8.1., VSD was marked with yellow arrow (TV: tricuspid valve, MV: mitral valve, RV: right ventricle, LV: left ventricle)

Besides the function on metabolism, FoxO proteins are also involved in regulating cells proliferation and apoptosis. Due to their function on regulating the cell cycle, apoptosis and DNA repair, FoxO proteins have long been considered to be the tumor suppressors (Brunet, Bonni et al. 1999; Medema, Kops et al. 2000; Kops, Dansen et al. 2002; Kops, Medema et al. 2002; Tang, Dowbenko et al. 2002; Tran, Brunet et al. 2002; Seoane, Le et al. 2004). The potential role of SMP5 in tumorigenesis through regulating FoxO proteins phosphorylation status is also a worthful investigation direction.

The expression of SMP5 is changing in mouse liver under different metabolism profile (Keller, Choi et al. 2008) which is further confirmed in my dissertation study by using western blotting (Figure 2.1). The varied protein level of SMP5 during fasting-feeding cycle suggests endogenous SMP5 is regulated and this regulation is highly related to metabolism homeostasis. Elucidating the mechanism through which SMP5 is regulated may enhance our understanding on blood glucose homeostasis and find new therapy target for diabetes.

In summary, we identified SMP5 as a FoxO1/3a phosphatase. SMP5 can interact and dephosphorylate FoxO1/3a *in vitro* and *in vivo*. Manipulating SMP5 expression in cells can mediate FoxO1/3a transcription activity through affect their subcellular localization (Figure 8.3). SMP5 knockout mouse has increasing phospho-FoxO1/3a in liver and decreasing gluconeogenesis related genes, PEPCK1 and G6PC, expression which associate with hypoglycemia. The finding of the gluconeogenesis related new

phosphatase may facilitate new therapeutic strategies developing for diabetes and glucose metabolism related disease.

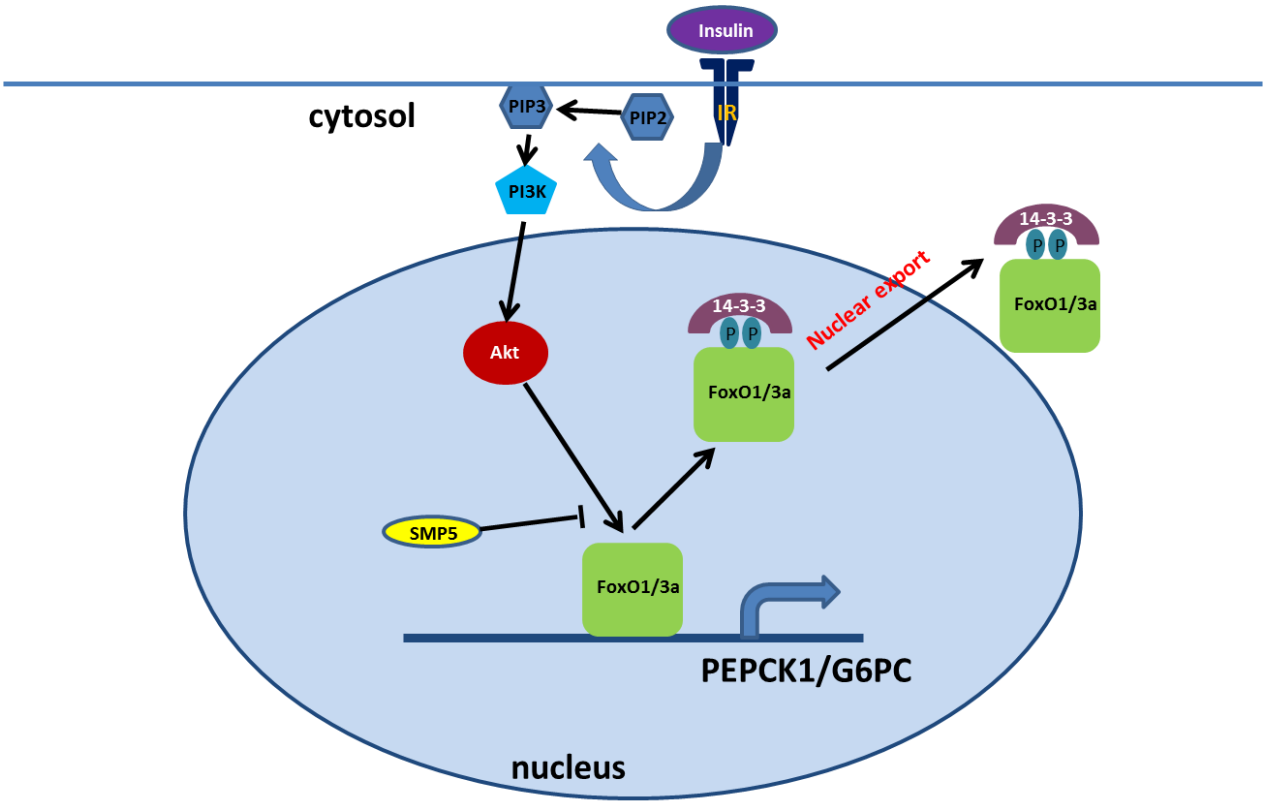


Figure 8.3 **The working model of SMP5 regulating FoxO1/3a transcription activity.** SMP5 facilitates FoxO1/3a nucleus sustaining through dephosphorylating FoxO1/3a and attenuating insulin signaling.

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