MODULATION OF THE ACTIVITY OF A KEY METABOLIC REGULATOR SMALL HETERODIMER PARTNER BY POST-TRANSLATIONAL MODIFICATIONS

BY

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DISSERTATION

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Abstract

Small Heterodimer Partner (SHP, NR0B2), a member of the nuclear receptor superfamily, is an orphan receptor that lacks a DNA binding domain but contains a putative ligand binding domain. SHP forms non-functional heterodimers with DNA binding transcriptional factors and, thereby, functions as a transcriptional corepressor in diverse biological processes, including cellular metabolism, cell proliferation, apoptosis, and sexual maturation. Of these reported functions of SHP, maintaining cholesterol and bile acid levels by negative feedback regulation of hepatic conversion of cholesterol to bile acids is well established.

Cholesterol is essential in many biological activities in mammalian cells. Conversion of hepatic cholesterol into bile acids is a major pathway to eliminate cholesterol from the body. However, excess amounts of cholesterol and bile acids are pathogenic. Therefore, the levels of cholesterol and bile acids need to be tightly regulated. Cholesterol 7α-hydroxylase (CYP7A1), a liver specific P450 enzyme, is the first and rate-limiting enzyme in this process. Increased levels of bile acids repress transcription of CYP7A1 in a feedback manner. In response to elevated bile acid levels, the nuclear bile acid receptor Farnesoid X Receptor (FXR) increases the transcription of SHP. SHP interacts with the hepatic DNA-binding activators, hepatic nuclear factor-4α (HNF-4α) or liver receptor homologue-1 (LRH-1) on the CYP7A1 promoter, and represses transcription of the CYP7A1 gene. In addition to regulating cholesterol and bile acid levels, SHP is known to mediate inhibition of fatty acid synthesis, hepatic lipogenesis, and glucose production in response to elevated bile acid levels.

Posttranslational modifications profoundly regulate protein stability and activity. Recently, bile acids have been reported to function as signaling molecules that activate kinase pathways. We recently found that SHP stability is increased by bile acid-activated ERK-

mediated phosphorylation through inhibition of ubiquitination. We now show that the activity of SHP is increased by posttranslational methylation of SHP at Arg-57 by protein arginine methyltransferase 5 in response to bile acids. The overall aim of this study is to delineate the molecular mechanism by which the post-translational modification of SHP regulates SHP functional activity.

In recent years, several naturally-occurring mutations in the SHP gene have been reported in human subjects that are associated with mild obesity and diabetes. About 30% of these reported mutations were Arg mutations, including the R57W mutation. Though it is known that the mutations lead to metabolic disorders, the molecular basis underlying the mechanism by which the mutations lead to metabolic disease is unknown. By mass spectrometry, we identified Arg 57 as a site of methylation in SHP catalyzed by Protein Arginine Methyltransferase 5 (PRMT5). Functional activity assays showed that methylation of SHP at Arg-57 by PRMT5 is important for SHP inhibition of LRH1 and HNF-4α transactivation.

Our lab previously showed the molecular mechanism of SHP-mediated repression involving the coordinate recruitment of chromatin modifying repressive cofactors, mSin3A/HDAC1, NCoR1/HDAC3, methyltransferase G9a, and the Swi/Snf-Brm remodeling complex, to the CYP7A1 promoter. Mutation of the Arg-57 site to Trp (R57W is the naturally-occurring mutant) decreased SHP interaction with corepressors that we had previously identified, and severely impaired inhibition of gene expression by SHP. Overexpression of wild type SHP in mouse liver resulted in decreased lipogenic, bile acid synthetic and gluconeogenic gene expression, and mutation of Arg-57 blocked SHP function, but remarkably in a gene-selective manner. Overexpression of the R57W mutant resulted in elevated levels of triglycerides and bile acids in liver compared to that of wild type SHP. Differential interaction and recruitment of

corepressors by SHP in a promoter-specific manner may contribute to gene-selective repression by the R57W mutant.

Our studies have shown that SHP is methylated by PRMT5 after bile acid treatment. Tandem mass spectrometry revealed that in addition to methylation at Arg-57, SHP is also phosphorylated at Thr-55 after bile acid treatment. Studies with kinase inhibitors showed that a signaling pathway involving PI3K and PKC ζ is involved in SHP Thr phosphorylation, and also regulates arginine methylation of SHP. The close proximity of the phosphorylation (Thr-55) and methylation (Arg-57) sites suggested a possible interplay between them. Studies with phosphorylation- and methylation-defective mutants demonstrated crosstalk between SHP Thr phosphorylation and Arg methylation.

This study demonstrates a critical role for Arg-57 methylation by PRMT5 in SHP function, and suggests a possible mechanism for association of the reported R57W mutation with obesity. This study also reveals Thr-55 phosphorylation of SHP by upstream kinase signaling pathways to be important for SHP functional activity. Targeting post-translational modifications of SHP may be an effective strategy to develop new therapeutic agents to treat SHP-related human diseases, such as metabolic syndrome, cancer, and infertility.

Dedication

To my late grandfather, my dear parents, brother Vamsi Krishna, husband Srivatsa Kalala and best friend Karuna Nelli

I wish to express my sincere gratitude to my advisor, Dr. Jongsook Kim Kemper, for her guidance and constant support throughout this work. She was always ready to provide her guidance and help in solving problems. This work would never have taken shape without her encouragement and expertise. I am also grateful to Dr. Byron Kemper for his valuable comments and support during the course of this work.

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Chapter One

Introduction

I. Nuclear receptor superfamily

A. Introduction

Nuclear receptors consist of a superfamily of ligand-regulated transcription factors that function as transcriptional switches in response to lipophilic signaling molecules including endocrine hormones, vitamins, xenobiotics and dietary lipids. They bind to specific DNA sequences and regulate the expression of target genes involved in almost every aspect of mammalian physiology (1, 2). Currently, 48 members have been identified in the human genome (3, 4) that can be broadly divided into three sub-groups based on their physiological ligands and potential functions (5). The first class of nuclear receptors is the classic endocrine receptors, the second class is the adopted orphan nuclear receptors and the third class includes true orphan nuclear receptors. The classic endocrine receptors are characterized by their very high affinity to ligands (Kd = nM range), and include steroid hormone receptors, such as the estrogen receptors (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR) and androgen receptor (AR) as well as thyroid hormone receptors (TR), and vitamin A (retinoid acid receptors, RAR) and D receptors (VDR). Orphan nuclear receptors are the subset of nuclear receptors identified based on their sequence homology to the endocrine nuclear receptors, which originally lacked a cognate ligand (6). Through reverse endocrinology, a receptor can be used to discover its natural ligand so that the receptor's potential function can be characterized (7). Many endogenous and exogenous compounds have been identified specifically for some orphans (8-12). These deorphanized nuclear receptors belong to the second class of nuclear receptors

called “adopted orphan nuclear receptors”, although for some adopted receptors, the role of ligand-dependent regulation mediated in physiology is not known. This class of nuclear receptors is characterized by a low affinity for their ligands (Kd = μM range). The third class of nuclear receptors comprises of true orphan nuclear receptors and includes all the remaining nuclear receptors for which ligands have not been identified yet.

A typical nuclear receptor is composed of four independent but interacting functional modules: a ligand-independent activation function modulator domain (AF-1), a central DNA binding domain (DBD) containing ~70 amino acids, a hinge domain and a C-terminal ligand binding domain (LBD), which contains a ligand-dependent activation function domain (AF-2) (Fig.1.2) (13-15). The AF-1 domain displays the most variability both in length and primary sequence and may regulate promoter-specific and cell-dependent activities. The DBD, which consists of two cysteine-rich zinc finger motifs, is highly conserved within the nuclear receptor family. The hinge domain is the region connecting the DBD and LBD, and is structurally flexible to allow the DBD to rotate 180^{o} which allow some receptors to bind as dimers to both direct and inverted hormone response elements (HREs). The less conserved LBD, which allows the binding of structurally diverse small lipophilic molecules in response to different signals, is responsible for ligand binding, dimerization and interaction with coactivators or corepressors (16-21).

Nuclear receptors can regulate transcription by binding to specific DNA sequences, referred to as hormone response elements (HREs). Nuclear receptors can bind to the DNA as monomers or as homodimers or heterodimers typically with Retinoid X Receptor (RXR) (22). Nuclear receptor transcriptional activity can be modulated through binding of ligands. Upon

ligand binding, the LBD of nuclear receptor undergoes a conformational change, which results in the release of corepressors and recruitment of coactivators to the AF-2, and facilitates gene transcription (23).

In the past two decades, numerous studies have shown that nuclear receptors play essential roles in many metabolic pathways, such as bile acid/cholesterol homeostasis, lipid/glucose metabolism, energy homeostasis, and inflammation (5, 24-30). Importantly, activity of nuclear receptors can be modulated by natural or synthetic ligands so that these pathways can be regulated. The critical physiological functions of nuclear receptors makes them potential targets for the treatment of metabolic diseases (31).

B. Orphan nuclear receptors

Historically, nuclear receptors were discovered by using ligands to "fish" for their receptors. These receptors were referred to as the classic hormone receptors. However with modern molecular biology techniques such as screening of cDNA libraries, it became possible to identify related receptors based on sequence similarity to known receptors without knowing what their ligands are, leading to the discovery of the orphan receptors.

Adopted orphan receptors in the nuclear receptor group include the farnesoid X receptor (FXR), liver X receptor (LXR), peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR), pregnane X receptor (PXR) and retinoid X receptor (RXR). Adopted orphans like FXR, LXR and PPAR bind metabolic intermediates such as bile acids, sterols and fatty acids, respectively, with relatively low affinity and hence function as metabolic sensors.

Others like CAR and PXR function as xenobiotic sensors up-regulating the expression of xenobiotic metabolizing enzymes like cytochrome P450 enzymes. RXR, whose ligand was identified as 9-cis-retinoic acid, associates with other nuclear receptors as a heterodimeric partner and participates in a wide range of nuclear receptor response systems (5, 7).

FXR acts as a sensor for bile acids, the end products of hepatic cholesterol catabolism. FXR is activated by primary bile acids, chenodeoxycholic acid (CDCA) and cholic acid (CA) and to a lesser extent by secondary bile acids, lithocolic acid (LCA) and deoxycholic acid (DCA). In the liver, FXR plays a pivotal role in maintaining bile acid, lipid and glucose homeostasis by regulating genes involved in these metabolic pathways. In response to elevated bile acids, bile acid activated FXR represses the transcription of the CYP7A1 gene, which is the first and rate-limiting enzyme in the bile acid synthetic pathway. This feedback repression is mediated by FXR induction of SHP gene expression (2, 7). SHP in turn binds to and inhibits the transactivation of hepatocyte nuclear factor 4α (HNF-4α) and liver receptor homologue 1 (LRH1) at the CYP7A1 promoter (Fig.1.1). FXR also protects the liver from elevated bile acids by controlling the expression of bile acid transporters, as well as fibroblast growth factors. It induces the expression of genes involved in bile acid efflux: bile salt export pump (BSEP), multidrug resistant-associated protein 2 (MRP2) and multidrug resistance P-glycoprotein 3 (MDR3), and represses the expression of Na^+-taurocholate cotransporting polypeptide (NTCP), which is involved in bile acid import. In addition, FXR promotes lipid clearance by inducing genes that regulate lipoprotein metabolism. The FXR/SHP pathway is also known to play a role in repression of the lipogenic gene, SREBP1c. The FXR/SHP cascade also regulates glucose homeostasis by repression of the gluconeogenic genes, PEPCK and G6pase.

True orphan nuclear receptors, whose ligands have not been identified, include SHP, DAX-1, Rev-erb, GCNF, TLX, PNR, COUP-TF, TR2, 4 and NR4A. SHP is an atypical orphan nuclear receptor since it lacks the conserved DBD and consists only of a putative LBD. Since its discovery, SHP has been identified as a key transcriptional repressor of genes involved in diverse metabolic pathways. The nuclear receptors repressed by SHP include LRH1, HNF4, ER, TR, estrogen-related receptor (ERR), LXR, FXR, RAR, RXR, PXR, AR, CAR and PPAR. Repression by SHP is not limited to nuclear receptors. A few other transcription factors that have been reported to interact with SHP include basic helix-loop-helix transcription factor (BETA2/NeuroD) and forkhead transcription factor (Foxo1). In addition, SHP mediates inhibition of transforming growth factor-beta (TGF-β).

SHP plays an important role in the negative feedback repression of bile acid biosynthesis by inhibition of the CYP7A1 gene, but is also involved in regulating the expression of genes with roles in bile acid transport, lipid metabolism, and gluconeogenesis. In addition, SHP induces apoptosis in liver and cancer cells, and also plays a role in cell proliferation, drug metabolism and energy homeostasis. In humans, mutations in the SHP gene are associated with mild obesity and diabetes. In SHP-null transgenic mice, bile acid pool size is elevated, energy expenditure is increased and pancreatic β cell function and glucose homeostasis are improved.

The important role of orphan nuclear receptors in metabolic regulation has been clearly established. Orphan nuclear receptors represent a unique and pivotal resource to elucidate new regulatory systems that impact both normal physiology and human disease. Although it is not

clear whether activity of orphan nuclear receptors can be pharmacologically modulated, further study on the mechanisms by which orphan nuclear receptor activity is regulated may uncover possible therapeutic strategies through modulating receptor function.

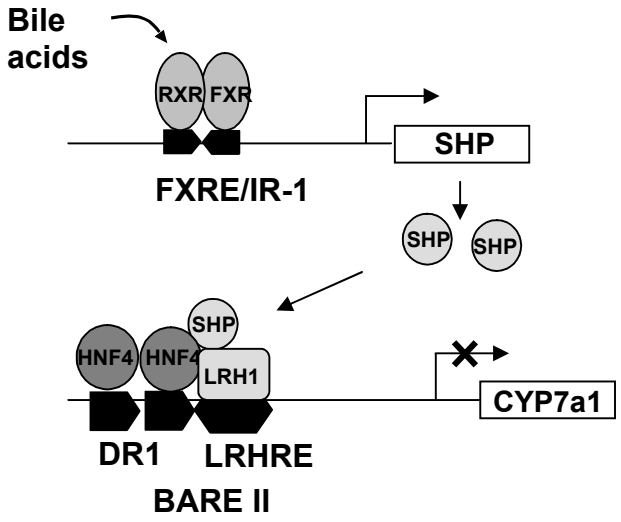

Fig. 1.1 Transcriptional repression of CYP7A1 by bile acids is mediated by nuclear receptors FXR and SHP. Bile acid response element II (BARE II) on the CYP7A1 promoter contains binding sites for nuclear receptors HNF-4α and LRH-1. The HNF-4α homodimer binds to the DR-1 motif, which has three nucleotides overlapping with the binding site for LRH-1. Both receptors serve as activators for CYP7A1. Bile acid activated FXR dimerizes with RXR and binds to the IR-1 motif on the SHP gene to increase SHP transcription. SHP protein in turn binds to the LRH-1/HNF-4 on the CYP7A1 promoter and represses its transcription.

II. SHP, an atypical orphan nuclear receptor

A. Atypical structure

SHP is an atypical orphan nuclear receptor that contains the dimerization and ligand-binding domain (LBD) found in other family members, but lacks the conserved DNA binding domain (DBD) (Fig. 1.2) (42-44). SHP was originally isolated in 1996 based on its interaction with

xenobiotic nuclear receptor CAR in a yeast two-hybrid screening (42). It is predominantly expressed in the liver and with lower levels in the intestine, heart, adrenal gland, and pancreas (42, 43). The ability of SHP to bind directly to a variety of NRs is crucial for its physiological function as a transcriptional inhibitor of gene expression. SHP binds to the AF-2 domain of nuclear receptors through two conserved functional LXXLL-related motifs (also called NR-boxes) located in the putative N-terminal helix 1 of the LBD and in the C-terminal region of helix 5 (45). These motifs are found in a variety of NR-binding proteins and are usually a characteristic site for binding of coactivators to the ligand-dependent AF-2 domain of nuclear receptors (43, 44, 46). They thus serve the same function in SHP as a binding site for other nuclear receptors (45). Therefore, SHP can compete with coactivators for binding to the AF-2 surface of nuclear receptors, implying a direct antagonism of coactivator function as an inhibitory mechanism of SHP (45). Studies also suggested that SHP possesses an intrinsic C-terminal repression domain, which has been proposed to recruit co-repressors (Co-R) to execute its active repression function (47). Deletion studies of SHP domains demonstrated that the intrinsic repression domain of SHP is required for its full inhibitory function (48, 49), implying the importance of active recruitment of corepressors in SHP-mediated repression. Several corepressors such as HDACs, mSin3A and NcoR have been reported to interact with SHP, supporting an active repressive mechanism for SHP (50).

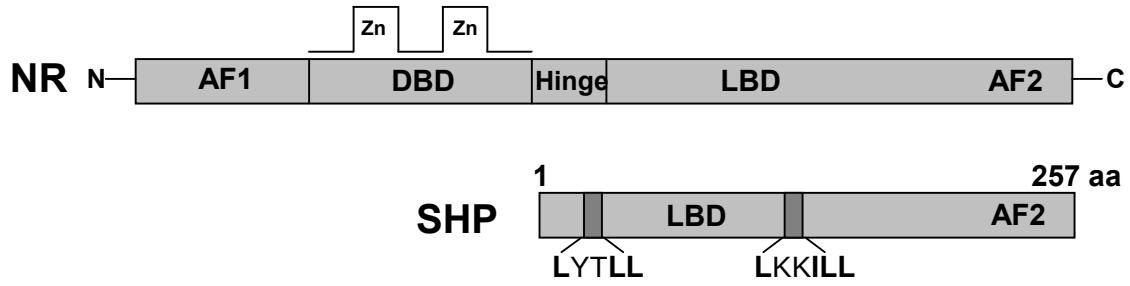

Fig. 1.2. Structure of a typical nuclear receptor and the atypical orphan nuclear receptor, SHP. A typical nuclear receptor (NR) contains an N-terminal activation function-1 (AF-1) domain, a conservative DNA binding domain (DBD), which is made up of two cysteine-rich zinc fingers and a ligand binding domain (LBD), which contains a ligand-dependent activation function-2 (AF-2) domain. However, SHP is an unusual nuclear receptor that lacks a DBD. It contains only a putative LBD and two LXXLL motifs for interaction with other nuclear receptors.

B. Molecular mechanisms of repression

Three distinct repression mechanisms have been suggested to explain the inhibitory function of SHP on the transcription of NR target genes: coactivator competition, active recruitment of corepressors, and inhibition of DNA-binding (51). The first mechanism of repression involves the binding of SHP to the AF-2 domain of NRs through two functional LXXLL-related motifs, which results in interference and direct competition with coactivator binding. This mode of inhibition is more pronounced in the case of SHP inhibition of transcription mediated by ERs, RXR, LRH-1, HNF4, AR, LXRs, ERRs, GRs and Nur77 (52). For example, SHP inhibits the interaction of PGC1α with LRH1 to inhibit CYP7a1 expression (53). In addition, it was shown that SHP antagonizes PGC1α coactivation of both HNF-4 and GR transactivation of the PEPCK promoter (54). SHP also interacts directly with agonist-bound ERα and ERβ, and inhibits ER-mediated transcriptional activation by targeting the ligand-regulated AF-2 and competing for binding of coactivators such as TIF2 (45, 46).

The second mode of repression suggested for SHP is the active recruitment of corepressors. Although coactivator competition on the AF-2 domain might be important for inhibition, recruitment of conventional corepressors could be critical for SHP to act as a direct transcriptional repressor since SHP contains a strong transcriptional repression domain at its C terminus. A few years ago, our lab demonstrated for the first time that SHP actively recruits an mSin3A/HDAC-1 histone deacetylase complex and a Swi/Snf-Brm chromatin remodeling complex to the native CYP7A1 promoter, which leads to reduced histone acetylation and a closed nucleosome structure on the CYP7A1 promoter, thus repressing the gene (55). SHP has also been shown to be associated with unmodified and lysine 9-methylated histone-H3 and to functionally interact with HDAC1 and the G9a methyltransferase, which led to histone deacetylation, followed by H3-K9 methylation and stable association of SHP itself with chromatin (56). Our lab also showed that G9a was recruited to the CYP7A1 promoter and H3K9 was methylated in a SHP-dependent manner (57). These studies established a critical role for G9a methyltransferase, histone deacetylases, and the Swi/Snf-Brm complex in the SHP-mediated inhibition of hepatic bile acid synthesis via coordinated chromatin modification at CYP7a1.

In addition, recently our group reported distinct functional specificities of Brm and Brg-1 Swi/Snf ATPases in the feedback regulation of hepatic bile acid biosynthesis. The Swi-Snf-Brm remodeling complex is involved in SHP-mediated repression of CYP7A1, and interestingly auto-repression of SHP, whereas the Swi/Snf-Brg-1 remodeling complex is involved in bile acid/FXR-mediated transcriptional activation of the SHP promoter. Brg-1, but not Brm, is a coactivator for FXR and is recruited to SHP promoter after bile acid treatment to enhance SHP transcription. In contrast, Brm, but not Brg-1, is involved in SHP-mediated repression of

CYP7a1 and auto-regulation (58). A recent interesting study showed that SHP recruited SIRT1, a class III histone deacetylase to LRH1 target gene promoters and SIRT1 deacetylated template-dependent histones H3 and H4 to inhibit transcription of LRH1 target genes (59).

Both of these modes of repression are involved in SHP inhibition of many NRs such as HNF4, ERs and LRH-1 (46, 47, 60). SHP might utilize these two distinct inhibitory steps in a cell type- and target gene-dependent manner.

The third possible mode of repression by SHP is SHP binding to a DNA bound NRs resulting in the dissociation of the SHP-NR complex from the promoters. For example, SHP has been shown to inhibit DNA binding and transcriptional activation by repressing RAR-RXR heterodimers, RAR-PXR heterodimers, and by repressing agonist dependent ERα dimerization and HNF4α homodimerization. HNF-3, JunD and C/EBPα were also reported to be repressed by SHP via inhibition of DNA binding (61-63).

C. Function of SHP as a regulator of diverse metabolic pathways

SHP was discovered only a decade ago, but increasing numbers of studies here demonstrated that SHP is a pleiotropic regulator, influencing multiple target genes involved in diverse biological processes (Fig. 1.3) (51, 64, 65).

The role of SHP in the negative feedback repression of bile acid biosynthesis through inhibition of CYP7a1 gene transcription has been very well studied. Many lines of evidence demonstrate that SHP also regulates several metabolic pathways involved in fatty liver and

obesity by acting as a transcriptional regulator of nuclear receptors in lipid homeostasis (95, 104). Bile acids have long been known to affect triglyceride (TG) homeostasis. In humans, bile acid-binding resins induce the production of VLDL TGs, whereas treatment of cholesterol gallstones with the bile acid CDCA has been shown to reduce hypertriglyceridemia (66). The mechanisms underlying this reciprocal relationship between bile acid biosynthesis and TG production has remained elusive. One hypothesis is that bile acids, by activating FXR, induce the expression of SHP. SHP then interferes with the expression of a key lipogenic gene, SREBP-1c, by inhibiting the activity of LXR and eventually other transcription factors that stimulate SREBP-1c expression (66). SREBP-1c controls the expression of other genes involved in lipogenesis including fatty acid synthase (FAS), acetyl CoA-carboxylase (ACC), acetyl-CoA synthetase (AceCS) and stearoyl-CoA desaturase-1 (SCD-1). Consistently, study by Matsukuma et al. showed that while LRH-1 stimulated FAS transcription via LXR, this response was blocked by increased SHP and that FAS mRNA was overexpressed in $SHP^{-/-}$ mice (67).

The study by Boulias et al. using SHP transgenic mice showed that the constitutive expression of SHP led to the depletion of hepatic bile acid pools, and accumulation of triglycerides in the liver resulting in a fatty liver phenotype (64). In SHP-transgenic mice, the mRNA levels of genes involved in fatty acid and triglyceride biosynthesis, such as SREBP-1c, FAS, ACL, ACC-1, and SCD1, and the fatty acid translocase gene, CD36 were increased significantly, which is in contrast to the study by Watanabe et al. (66). The proposed mechanism for the upregulation of these genes was the indirect activation of LXRα (which activates SREBP-1c gene), and PPARγ (which activates CD36) by SHP, probably by the action of SHP on cholesterol catabolic enzymes (64).

A second possibility is that bile acids may lower TG synthesis by activating signaling pathways that leads to post-translational modifications of SHP, FXR or other nuclear receptors that modulates their activity. Recently, bile acids have been identified as signaling molecules that activate kinase cascades, such as mitogen-activated protein kinases (MAPKs) (68-70) , and growth factor receptors, as well as cell surface receptors such as G-protein-coupled receptors (71, 72). My study has demonstrated that bile acid activated kinase signaling pathways may increase post-translational modification of SHP, which increases the repressive activity of SHP. Detailed findings will be presented in Chapters 2 and 3.

Several studies have shown that SHP has a major function in regulating hepatic gluconeogenesis. Increased bile acids inhibit the expression of the gluconeogenic genes, G6Pase, PEPCK, and fructose 1, 6-bis phosphatase (FBP1), in a SHP-dependent manner and the absence of this repression in both FXR−/− and SHP−/− mice indicates that FXR-SHP nuclear receptor cascade is critical for regulating glucose metabolism (73, 74). AMP-activated protein kinase (AMPK) is a serine/threonine kinase that regulates hepatic glucose and lipid homeostasis by affecting a diverse set of target genes associated with these metabolic pathways. Metformin, an antidiabetic drug widely used for the treatment of type 2 diabetes, and sodium arsenite which was previously reported to exhibit insulin-mimetic effects on glucose homeostasis have been reported to inhibit hepatic gluconeogenesis in an AMPK-dependent manner through SHP-mediated inhibition of PEPCK and G6Pase gene expression (75, 76). These studies provide a novel molecular mechanism of SHP mediated regulation of hepatic glucose homeostasis and indicate that SHP may be one of the primary targets of AMPK.

SHP inhibits the GR-mediated activation of PEPCK promoter by antagonizing interaction of PGC1α with GR. SHP also represses the PEPCK and G6Pase gene expression via inhibition of the forkhead transcription factors HNF-3 and HNF-6. It was also shown that SHP directly interacts with C/EBPalpha on the PEPCK promoter and inhibits its transcription. Other important targets of SHP in glucose metabolism are the forkhead transcription factor FOXO1, the basic helix-loop-helix transcriptional factor BETA2/NeuroD, and the aryl hydrocarbon receptor (AHR)/nuclear translocator (ARNT).

Recent studies have shown that SHP also plays an important role in regulation of the transcription of several microRNAs (77-80). A recent study in our lab showed that FXR induces expression of SHP, which in turn blocks the occupancy of p53 at the miR-34a gene promoter and represses miR-34a expression, which in turn leads to the positive regulation of the NAD-dependent deacetylase SIRT1 in the liver (81). This study demonstrated that the FXR/SHP pathway controls SIRT1 levels via miR-34a inhibition and that elevated miR-34a levels in obese mice due to defective FXR/SHP pathway contributes to decreased SIRT1 levels observed in these mice.

Although most studies have reported that SHP acts as a repressor of gene transcription, SHP has also been found to activate the nuclear factor-kappa B (NF-κB) in resting macrophage cells treated with oxidized low density lipoprotein (oxLDL) (82). Moreover, SHP was also reported to upregulate the transcriptional activity of PPARγ by directly binding to the DBD/hinge region of PPARγ (83).

All these findings imply that SHP is as an integrative regulator of diverse arrays of biological activities (66, 84). SHP gene mutants in humans have been reported to be associated with obesity and diabetes (85-90). SHP null mice showed increased bile acid pool size due to impaired feed back repression of bile acid biosynthesis (91, 92). In addition to impaired bile acid homeostasis, SHP-/- mice are also resistant to high-fat diet-induced obesity (93, 94). Ob/ob mice (mice with deletion of Leptin gene) exhibit elevated SHP expression (93). However SHP and Leptin double knockout mice do not exhibit the fatty liver observed in ob/ob mice (93). In line with this, in transgenic mice over-expressing SHP bile acid pools are depleted and hepatic triglycerides are accumulated (64). These results strongly indicate that SHP plays an important role in regulating bile acid/cholesterol and lipid homeostasis. In addition, SHP -/- mice also exhibited hypoinsulinemia, which was connected with increased insulin sensitivity (84). SHP deletion also causes a transformed phenotype of mouse embryonic fibroblasts and a spontaneous hepatic tumor formation was observed, implying that SHP functions as a tumor suppressor (95, 96). These data demonstrate that SHP is also involved in glucose homeostasis and carcinogenesis. Recent work has shown that SHP may also play a role in macrophages. The expression of SHP along with target nuclear receptors during macrophage activation suggested an involvement in atherogenesis and inflammatory disease. As SHP plays a role in diverse cellular pathways, targeting SHP activity could serve as a potential therapeutic approach for treating several metabolic diseases.

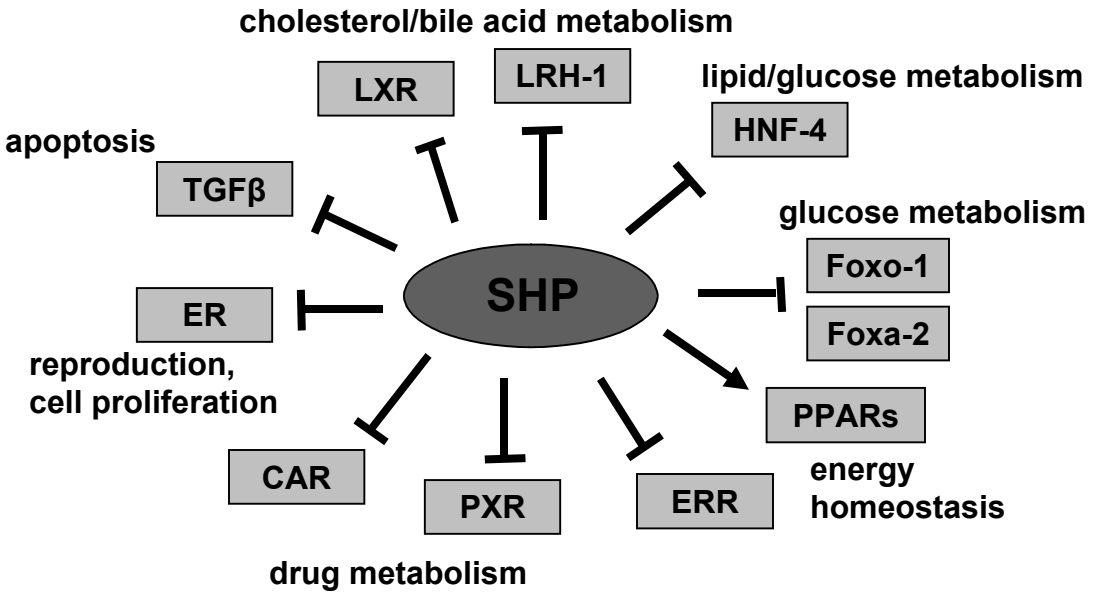

Fig. 1.3. Potential functions of SHP in diverse biological processes. SHP interacts with and inhibits the activities of numerous nuclear receptors and transcription factors as indicated, involved in metabolic pathways, cell cycle control and energy homeostasis including ER, HNF-4, LRH-1, LXR, CAR, PXR, ERRs, PPARs, Foxo-1, Foxa-2, p53 and TGFβ.

D. Function of SHP in maintaining bile acid/cholesterol homeostasis

In humans, cholesterol is acquired through dietary absorption and de novo biosynthesis from acetyl-CoA. The elimination of cholesterol from the body through catabolization into bile acids is the major pathway to eliminate excess cholesterol from the body (11, 14). Biosynthesis of bile acids generates bile flow from the liver to the intestine, and 95% of bile acids are efficiently reabsorbed into the portal venous system, and transported back into the liver (14). This enterohepatic circulation of bile is important for maintaining liver function and regulating metabolic pathways. Bile acids are amphipathic molecules that function as physiological detergents to facilitate absorption, excretion and transport of lipids, cholesterol as well as fat-soluble nutrients such as vitamin D and E, and metabolites in the liver and intestine (16-18).

Bile acid synthesis is tightly regulated under normal physiological conditions. CYP7A1, which is exclusively expressed in the liver, is the first and rate-limiting enzyme in the classic pathway; therefore the output of bile acids largely depends on the regulation of CYP7A1. In the presence of elevated bile acid levels in the body, CYP7A1 is predominately regulated at the transcriptional level by bile acids in a feedback manner (22, 31, 32, 68, 97). Regions containing bile acid response element (BARE) I and II were identified in the CYP7A1 promoter (32, 68, 98). The identified BARE II in the human CYP7A1 promoter contains binding sites for nuclear receptors HNF-4α and LRH-1, which have three overlapping nucleotides (32, 68, 99). Both HNF4α and LRH-1 serve as DNA-bound transcriptional activators in the regulation of CYP7A1 (100, 101). In 2000, two independent research groups reported an elegant cascade pathway in bile acid-mediated repression of CYP7A1 transcription, in which SHP plays a critical role (53, 102). FXR has been identified as a receptor for a wide variety of endogenous bile acids through NRs/ligand binding assays (104, 22). Like other adopted orphan nuclear receptors, FXR forms a permissive heterodimer with (RXR) and binds to specific FXR-responsive DNA elements (FXREs), IR-1 motifs, on SHP promoter and increases SHP gene transcription (53, 102, 105). Induced SHP protein in turn binds to LRH-1 on the BARE II on CYP7A1 promoter to inhibit the transcription of CYP7A1 (Fig. 1.1) (53, 102). The molecular basis for SHP interaction with LRH-1 to regulate cholesterol and bile acid homeostasis has been demonstrated in a recent structural and biochemical study (106). Mice lacking SHP gene failed to repress CYP7A1 expression in response to a FXR synthetic agonist GW4064 (107). SHP null mice also exhibited two-fold higher amounts of accumulated serum bile acid compared to wild type mice (107, 108). These data demonstrate that SHP plays a crucial role in the regulation of CYP7A1 gene and thus is important in maintaining bile acid/cholesterol homeostasis.

III. Bile acids as signaling molecules

A. Functions of bile acids

Bile acids are synthesized from cholesterol in the liver and stored in the gall bladder as the main constituent of bile. After ingestion of food, bile flows into the duodenum, where it facilitates the solubilization and digestion of lipid-soluble nutrients and metabolites (16-18). Besides the role that bile acids play as physiological detergents, they have been implicated as versatile signaling molecules with endocrine functions. Bile acids have been shown to activate specific nuclear receptors including FXR, PXR and VDR, G protein coupled receptors (GPCRs) such as TGR5 (71, 72), and cell signaling pathways including the three mitogen-activated protein kinase (MAPK) signaling pathways (ERK, JNK and p38 MAPK), PKA, PKC and AKT/PKB (68-70). Through activation of these diverse signaling pathways, bile acids have been shown to regulate triglyceride, cholesterol, glucose and energy homeostasis (109-112).

B. Bile acids as ligands for FXR

FXR has recently been identified as a nuclear receptor for bile acids. CDCA, DCA and LCA are endogenous ligands for FXR in decreasing order of potency. FXR/RXR, activated by binding to bile acids, induces the expression of SHP, which induces the repression of the bile acid biosynthetic genes, CYP7A1 and CYP8B1. Activation of FXR also leads to increased expression of intestinal bile acid binding protein (I-BABP) and basolateral bile acid transporters (organic solute transporters – OSTα and OSTβ) in the intestine that may be involved in trafficking of bile acids during enterohepatic circulation. FXR also increases the expression of fibroblast growth factor 19 (FGF19). FGF19 signals from the intestine to the liver by binding to cell-surface FGF

receptor 4 (FGFR4) and leads to the repression of CYP7A1 expression via a JNK-dependent mechanism (113, 114).

C. Major kinase signaling pathways activated by bile acids

Bile acids have been shown to activate multiple kinase signaling pathways including MAPKs, protein kinase A (PKA), protein kinase C (PKC) and AKT/ protein kinase B (PKB) (68-70). MAPKs are a family of serine/threonine kinases that play important roles in response to changes in the cellular environment (115-118). The MAPKs include extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1/2/3), p38 and ERK5 (116, 117). Bile acids activate the JNK1/2 signaling cascade primarily by the FXR-dependent synthesis of FGF15/19 in the intestine. The secreted FGF15/19 hormone is transported to the liver, where it binds to FGFR4 and activates JNK1/2, which downregulates CYP7A1 gene transcription. It was postulated that this effect could be through JNK-mediated phosphorylation of HNF4α, which reduces its transcriptional activity. It has also been reported that bile acids upregulate SHP transcription through activating JNK kinase. Activated c-Jun can bind to its response element, activator protein-1 (AP1) site, on the SHP promoter and increase SHP gene transcription (68). Bile acids can also activate AKT (insulin signaling pathway) in hepatocytes. Activation of the AKT pathway allows bile acids to function in a manner identical to insulin in regulating glucose metabolism in the liver. Bile acids have been shown to differentially activate PKC isoforms in several cell types including hepatocytes, fibroblasts, colonic epithelial cells and kidney cells. Effects of bile acids on the translocation and activation of PKC isoforms has been well studied (119, 120). However, further studies are needed to identify downstream consequences of PKC

activation and understand whether such effects contribute to various diseases including diabetes, cancer and hypercholesterolemia.

D. Bile acid signaling in lipid, glucose and energy homeostasis

Bile acids function as signaling molecules not only for the feedback inhibition of their own synthesis, but also to regulate lipid and TG, glucose and energy homeostasis. Bile acids activate FXR, which in turn increases SHP expression. SHP causes feedback regulation of hepatic fatty acid and TG biosynthesis as well as VLDL production. SHP interferes with SREBP-1c expression, which in turn leads to reduced expression of genes downstream of SREBP-1c including FAS, ACC, AceCS and SCD1 (66). FXR activation also leads to increased expression of PPARα, which promotes fatty acid oxidation, the VLDL receptor that promotes TG clearance and ApoCII, which coactivates lipoprotein lipase, and to decreased expression of ApoCIII, which inhibits lipoprotein lipase.

Bile acids affect glucose metabolism by at least two mechanisms. First, bile acids activate SHP expression via FXR. SHP binds to FOXO1, C/EBPα, and HNF4α, transcription factors that activate the gluconeogenic genes, PEPCK and G6pase, and supresses gluconeogenesis (63, 73, 110, 74). Therefore, overexpression of SHP or activation of FXR by its agonists leads to decreased hepatic PEPCK and G6Pase gene expression. In line with this, $FXR^{-/-}$ mice show impaired glucose tolerance and insulin sensitivity. Secondly, through an FXR-independent manner, conjugated bile acids activate the phosphoinositide 3-kinase (PI3K)-AKT pathway (insulin signaling pathway) via G-protein coupled receptors or superoxide ions and function much like insulin to activate glycogen synthase and repress gluconeogenic genes.

Bile acids also have effects on energy homeostasis. Administration of bile acids to mice increases energy expenditure in brown adipose tissue (BAT), preventing obesity and insulin resistance (112). This effect is mediated by increased cAMP production, which stems from the binding of bile acids with the G protein coupled receptor TGR5. This is supported by the observation that $TGR5^{-/-}$ female mice are predisposed to obesity when fed a high-fat diet, and $TGR5^{-/-}$ male mice show a tendency for weight gain (122). It has also been shown that the bile acid activated FXR target SHP inhibits PGC1α expression and energy production in BAT, as concluded from the resistance of $SHP^{-/-}$ mice to diet-induced obesity (121).

IV. Signal-dependent regulation of SHP activity by post-translational modification

A. SHP post-translational modifications

Post-translational modification (PTM) is the chemical modification of a protein after its translation, such as acetylation, phosphorylation, methylation, ubiquitination and SUMOylation (SUMO = small ubiquitin-like modifier). PTMs of histones have been extensively studied and established as a major regulatory mechanism for eukaryotic gene transcriptional regulation (123, 124). The combination of histone PTMs is known as a histone code (125). With the emergence of new techniques, it is becoming evident that PTMs happen at high density in a variety of proteins. For example, recent phosphoproteomic analyses revealed that majority of proteins are phosphorylated in mammalian cells at one or more sites (126). With the awareness of the existence of PTMs of non-histone proteins, an increasing number of non-histone proteins have been reported as post-translationally modified, including nuclear receptors and their coregulators,

such as FXR, RXR, LRH-1, and PPARγ, and coregulators such as HDAC-1 and SRC-3 (126-134). The functional studies of PTMs revealed that PTMs can dynamically and remarkably regulate stability, cellular localization and function of transcriptional factors (127, 128, 132-136).

A single PTM may modulate protein activity or stability possibly by altering protein-protein interactions. However, many proteins exhibit more than one form of modification (131, 132). The combinations formed by these modifications may either positively or negatively cross-talk with each other (137). For example, SUMOylation of Mdm2 and HIF-1 at a Lys blocks ubiquitination of the same Lys site, thereby enhancing protein stability and function (138, 139). In the case of Cyclin D and 4E-BP1, phosphorylation of the proteins promotes their ubiquitination, thus decreases their protein stability (140, 141)

Since PTMs can profoundly affect protein activity, and bile acids can function as signaling molecules, the possibility that in addition to increasing SHP induction, bile acid signaling can also result in SHP PTMs was examined. The differential modification of SHP after bile acid treatment may contribute to its altered inhibitory activity by modulating (1) interaction with corepressors, (2) stability, and (3) cellular localization. It has been already reported by our group that bile acids increase SHP stability by activating ERK-mediated SHP phosphorylation at Ser-26 and inhibiting ubiquitination at Lys-122 and Lys-123 sites (142). In my study, I examined whether bile acids regulate SHP activity through other PTMs of SHP. My data demonstrate that in addition to increasing stability, bile acids can also activate other PTMs of SHP, which increase interaction with corepressors, ultimately increasing SHP functional activity. Detailed findings are presented in Chapters 2 and 3.

B. Bile acids and FGF19 activate signaling kinase pathways that activate SHP PTM

The emergence of bile acids as signaling molecules has led not only to the identification of novel signaling networks, but also to the understanding of the mechanisms of transcriptional regulation of metabolic pathways. The discovery of the bile acid receptor FXR (8, 36, 106) represented an important milestone in the definition of the mechanism of feedback mediated by bile acids on *CYP7A1* transcription. Activation of FXR by bile acids represses CYP7a1 transcription in two ways, one by increasing SHP transcription in the liver, second by increasing FGF15/19 transcription in the intestine (114). It has been reported that FGF15/19 significantly represses CYP7A mRNA levels, and the repression is dramatically impaired in SHP-null mice, indicating that the repression is largely dependent on SHP. However, surprisingly, FGF15/19 did not increase SHP mRNA levels in mice (114). This study clearly demonstrated that FGF15/19 suppresses CYP7A1 transcription in a SHP-dependent manner, but without inducing SHP gene transcription. Therefore, the possibility that in addition to increasing SHP induction, bile acid and/or FGF15/19 signaling can also result in SHP PTMs that affect SHP activity rather than gene expression was examined and recently, our group reported that bile acid and FGF19 signaling pathways activate ERK-mediated SHP phosphorylation at Ser-26, which inhibits ubiquitination at Lys-122 and Lys-123 sites (142). Phosphorylation at Ser-26 leads to increased stability of SHP, thereby increasing the repression of CYP7A1 by SHP. This raises the question whether bile acid and FGF15/19 signaling pathways could activate other PTMS of SHP that affect its activity. Study presented in Chapters 2 and 3 address this question.

The bile-acid- and FGF15/19-controlled signaling pathways provide promising novel drug targets to develop novel therapeutic and preventative strategies that are useful in the clinical management of obesity, type 2 diabetes, hyperlipidaemia and atherosclerosis.

C. Naturally-occurring mutations in SHP gene associated with obesity and diabetes

There have been several reports that mutations in the gene encoding SHP are associated with early-onset obesity and high birth weight in Japanese, and to a lesser extent, in European population (85-89). Probands with SHP mutations had birth weights at least 1 standard deviation higher than the mean birth weight adjusted for gestational age in population-based control subjects and showed hyperinsulinemia and decreased insulin sensitivity, suggesting a possible physiological mechanism for the observed effects on birth weight and adiposity. Based on the study of SHP mutations in Japanese subjects, the authors speculate that SHP mutations could be a component of the genetic background of obesity in Japanese, although neither the significance of such mutations in the development of adult-onset obesity in this population nor the prevalence of mutations in western populations is known very well (85, 86). Also, a gender-dependent effect on penetrance for SHP deficiency was found in obese Chinese pedigrees (90). It was observed that the penetrance of male loss-of-function mutation carriers was significantly lower than that of female loss-of-function mutation carriers, suggesting that other genetic and/or environmental factors can modify the effects of SHP. Furthermore, it was demonstrated that an increased risk for type 2 diabetes was associated with SHP gene mutations (86).

Nuclear receptors such as SHP and PPAR α that regulate lipid metabolism in liver are potential contributors to fatty liver. Further, the storage of lipids in liver can trigger inter-organ

crosstalk that affects insulin sensitivity in muscle. FXR-null mice, with reduced levels of SHP, develop severe fatty liver and elevated circulating FFAs, which is associated with elevated serum glucose and impaired glucose and insulin tolerance resulting from attenuated inhibition of hepatic glucose production by insulin and reduced peripheral glucose disposal (74). Some patients with *SHP* mutations exhibit liver dysfunction due to fatty liver (85). Accordingly, mutations in SHP may be associated with insulin resistance due to both later obesity and also to fatty liver in Japanese subjects.

Though there have been several reports that the SHP mutations cause metabolic disorders, the molecular mechanisms by which the SHP mutations cause these disorders are unknown. The precise mechanism underlying the effect of the SHP protein on metabolic regulation has not yet been established. Many of the reported target factors for SHP repression are implicated in regulating gene expression in liver and pancreatic β-cells, including hepatic nuclear factor-4α (HNF4α), LRH1, Foxa2 and Neuro D, resulting in increased insulin secretion. A loss of SHP action should lead to increased activity of HNF4α, LRH1, Neuro D and Foxa2, resulting in increased expression of their target genes, which might lead to metabolic diseases (143). In this thesis, I have examined the molecular mechanism by which one of the SHP mutations reported in Japanese subjects, the R57W mutation, causes metabolic disease state, which will be discussed in detail in Chapter 2.

D. Protein arginine methyltranferase enzymes (PRMTs) and Arginine methylation: Regulation of SHP activity by PRMT5

Regulation of specific gene transcription by endocrine signals usually involves altered recruitment of transcriptional regulator proteins to the promoter, enhancer or silencer regions of target genes or alteration of the activity of proteins already associated with the gene. Frequently, these two mechanisms of gene regulation are accomplished by specific PTM of the proteins involved in transcriptional regulation. Such modifications alter protein function in specific ways. The roles of phosphorylation and acetylation in transcriptional regulation have been extensively studied, but recently the importance of other types of protein modifications, including methylation and sumoylation, have begun to be recognized (144).

Protein methylation is one of the most frequent protein modifications. About 2% of arginine residues were found to be dimethylated in total protein extracts from rat liver nuclei (145). Protein arginine methyl transferases (PRMTs) are enzymes that catalyze transfer of methyl groups from S-adenosyl methionine to the guanidino nitrogen of arginine (144, 146-148). Eight mammalian protein arginine methyltransferase (PRMT) family members have been identified. They fall into two predominant classes based on the types of methylarginine products they produce (148). Type I enzymes (PRMT1, PRMT3, PRMT4/CARM1, PRMT6, and PRMT8) form monomethylarginine and asymmetric dimethylarginine, and type II enzymes (PRMT5, PRMT7, and FBXO11) form monomethylarginine and symmetric dimethylarginine. No activity has yet been demonstrated for PRMT2 and PRMT9 (144, 145). Cellular processes regulated by arginine methylation include RNA processing, transcriptional regulation, signal transduction and DNA repair (144, 149).

PRMT5 is a type II enzyme that methylates non-histone proteins as well as histones (144, 146). PRMT5 acts as a transcriptional repressor by methylating histones H3 and H4 and

transcriptional elongation factor SPT5 (150, 151). Recent studies have shown that PRMT5 plays an essential role in Brg1-dependent chromatin remodeling and gene activation during myogenesis (152) and that PRMT5 is required for early-gene expression in the temporal control of myogenesis (153). Arg methylation of Piwi proteins also plays an important role in the small noncoding piRNA pathway in germ cells (154). PRMT5 was recently shown to regulate the function of p53 in response to DNA damage by catalyzing Arg methylation (155). However, functional roles of PRMT5 as an important transcriptional coregulator of metabolic pathways have not been reported. In Chapter 2, we show that post-translational methylation by PRMT5 is critical for SHP function. In response to bile acid activated kinase signaling pathways, SHP is post-translationally methylated by PRMT5 at Arg-57, which is a naturally-occurring mutation (R57W) reported among Japanese subjects with obesity.

E. SHP as a potential therapeutic target for treating metabolic syndrome

SHP is a key metabolic regulator that regulates diverse metabolic pathways. The critical role of SHP in not only the feedback inhibition of bile acid synthesis, but also lipogenesis, gluconeogenesis and energy homeostasis has been clearly demonstrated. SHP null mice show increased bile acid pool size due to impaired feed back repression of bile acid biosynthesis (91, 92). In addition, SHP-/- mice are also resistant to high-fat diet-induced obesity (93, 94). Genetic variations in the SHP gene are associated with mild obesity and high birth weight in humans (85-90). Although SHP is an orphan NR, its conserved ligand-binding domain suggests the existence of SHP ligands. Bile acids activate SHP phosphorylation that increases its stability. Likewise, additional SHP PTMs that may increase its activity were also examined in my studies. Targeting SHP PTMs may provide a useful tool for determining the therapeutic value of SHP in treating

metabolic disease. The pharmacological manipulation of SHP function may serve as a potential therapeutic approach in preventing and treating diseases associated with metabolic syndrome (ex. diabetes, cholestasis, obesity), inflammatory processes (ex. atherogenesis, infections) and cell proliferation (ex. breast cancer).

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Chapter Two

Arginine methylation by PRMT5 at a naturally-occurring mutation site is critical for liver metabolic regulation by Small Heterodimer Partner

Abstract

Small Heterodimer Partner (SHP) inhibits numerous transcription factors that are involved in diverse biological processes, including regulation of bile acid metabolism. In response to increased hepatic bile acids, SHP gene expression is induced by the bile acid receptor, FXR, and the SHP protein is stabilized. We now show that the activity of SHP is increased by posttranslational methylation of SHP at Arg-57 by protein arginine methyltransferase in response to bile acids. Hepatic depletion of PRMT5 decreased SHP methylation and reversed the suppression of metabolic genes by SHP. With the exception of G9a methyltransferase, mutation of Arg-57 decreased the interaction of SHP with its known chromatin modifying repressive cofactors and decreased their recruitment to SHP target genes. Adenovirus-mediated hepatic expression of SHP in mice repressed metabolic target genes and mutation of Arg-57 reversed the inhibition in a gene-selective manner. The importance of Arg-57 methylation for the repression activity of SHP provides a molecular basis for the observation that a natural mutation of Arg-57 in humans is associated with the metabolic syndrome. Targeting post-translational modifications of SHP may be an effective strategy to develop new therapeutic agents to treat SHP-related human diseases, such as metabolic syndrome, cancer, and infertility.

Introduction

Small Heterodimer Partner (SHP, NR0B2), was discovered as a unique member of the nuclear receptor superfamily that lacks a DNA binding domain but contains a putative ligand binding domain (32). SHP forms non-functional heterodimers with DNA binding transcriptional factors including nuclear receptors and, thereby, acts as a transcriptional corepressor in diverse biological processes, including metabolism, cell proliferation, apoptosis, and sexual maturation (1, 3, 11, 35, 36, 39). Well studied hepatic functions of SHP are the inhibition of bile acid biosynthesis, fatty acid synthesis, and glucose production in response to bile acid signaling (1, 3, 4, 12, 19, 22, 37, 38). We previously showed that SHP inhibits expression of a key bile acid biosynthetic gene, CYP7A1, by coordinately recruiting chromatin modifying repressive cofactors, mSin3A/HDAC1, NCoR1/HDAC3, methyltransferase G9a, and the Swi/Snf-Brm remodeling complex, to the CYP7A1 promoter (9, 16, 25). GPS2, a subunit of the NcoR1 corepressor complex, was recently shown to act as a SHP cofactor and participates in differential regulation of bile acid biosynthetic genes, CYP7A1 and CYP8B1 (31).

Consistent with its important functions in metabolic pathways, naturally-occurring heterozygous mutations in the SHP gene have been associated with human metabolic disorders (7, 8, 27). About 30% of these reported mutations occur at arginine (Arg) residues, implying that functionally relevant posttranslational modification (PTM) at these sites may be important for SHP function. In response to elevated hepatic bile acid levels, SHP gene induction by the nuclear bile acid receptor FXR has been established (12, 22). We recently found that SHP undergoes a rapid degradation in hepatocytes and that SHP stability is increased by bile acid-activated ERK-mediated phosphorylation which inhibits its ubiquitination (26). In addition to

these changes in the levels of SHP, it is possible that the repression activity of SHP is also regulated in response to elevated hepatic bile acid levels.

Protein arginine methyl transferases (PRMTs) are enzymes that catalyze transfer of methyl groups from S-adenosyl methionine to the guanidino nitrogen of Arg (2, 21). Type I or type II PRMTs also catalyzes asymmetric or symmetric dimethylation of Arg, respectively. Both types of PRMTs also catalyze monomethylation of Arg. PRMT5 is a type II enzyme that methylates non-histone proteins as well as histones (2, 21). PRMT5 acts as a transcriptional repressor by methylating histone H3 and H4 and transcriptional elongation factor SPT5 (20, 28). Recent studies have shown that PRMT5 plays an essential role in Brg1-dependent chromatin remodeling and gene activation during myogenesis (6) and that PRMT5 is required for early-gene expression in the temporal control of myogenesis (5). Arg methylation of Piwi proteins also plays an important role in the small noncoding piRNA pathway in germ cells (34). PRMT5 was recently shown to regulate the function of p53 in response to DNA damage by catalyzing Arg methylation (15). However, functional roles of PRMT5 as an important transcriptional coregulator of metabolic pathways have not been reported.

Using molecular, cellular and in vivo mouse studies, we demonstrate that post-translational methylation by PRMT5 enhances SHP activity in response to bile acid signaling. PRMT5 methylated SHP at Arg-57, which is a sie for a naturally-occurring mutation associated with the metabolic syndrome in humans (7, 8, 27).

Materials and Methods

Materials and Reagents

Antibodies for SHP (sc30169), lamin A (sc-20680), tubulin (sc-8035), HDAC1 (sc-7872), mSin3A (sc-994), Brm (sc6450), LRH1 (sc-5995 X), PolII (sc-9001) and GFP (sc-8334) were purchased from Santa Cruz Biotech, M2 antibody was from Sigma and antibodies for PRMT5, G9a, and dimethyl symmetric Arg (SYM10) were purchased from Upstate Biotech. Purified recombinant PRMT5 protein was purchased from Abnova.

Construction of plasmids and adenoviral vectors

The expression plasmids, pcDNA3 flag-R57W and R57K mutants were generated using QuikChange site-directed mutagenesis kit (Stratagene) and positive clones were identified by DNA sequencing. For constructing Ad-flag-human SHP wild type and R57W mutant adenoviral vectors, the 0.9 kb fragment from pCDNA3-flagSHP plasmid was inserted into Xba1-digested Ad-Track-CMV vector. For Ad-siPRMT5 construction, siRNA sequences for PRMT5 were used as described previously (27). Annealed siRNA oligonucleotides were inserted into BamH1/HindIII sites of the pRNATin-H1.2/Hygro vector. A 4.5 Kb fragment with the H1 promoter and siRNA oligos was cut from pRNATin-siPRMT5 and inserted into the BglII/HindIII sites of Ad-Track-vector.

Cell cultures and transfection reporter assay

HepG2 cells (ATCC HB8065) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium. Cos-1 cells were maintained in DMEM medium. Cells were transfected with plasmids or infected with adenoviral vectors, incubated with serum-free media overnight, and treated with 50 μM CDCA for indicated times in figure legends.

In vivo experiments

BALB/c male mice were injected into the tail vein with Ad-flag-SHP, control Ad-empty, Ad-siPRMT5, or control scrambled RNA (0.5-1.0x10^9 active viral particles in 200 μl PBS). Five to seven days after infection, mice were fed normal or 0.5 % CA supplemented chow for 3 h starting at 5 PM and tissues were collected at 8 PM for further analysis. Feeding mice with CA chow for 3h increased Shp mRNA levels and decreased Cyp7a1 mRNA levels (25). For in vivo methylation assays, flag-SHP was immunoprecipitated under stringent conditions with SDS-containing RIPA buffer and methylated SHP at Arg was detected by western analysis using SYM10 antibody. All animal use and adenoviral protocols were approved by the Institutional Animal Care and Use and Institutional Biosafety Committees at University of Illinois at Urbana-Champaign and were in accordance with National Institutes of Health guidelines.

Measurement of bile acid pool and liver triglyceride levels

The bile acid pool from the gall bladder, liver and small intestine was measured by colorimetric analysis (Trinity Biotech). Liver triglyceride levels were measured using Sigma kit TR0100 according to the manufacturer's instruction.

Glucose and insulin tolerance tests

BALB/c male mice were injected into the tail vein with control Ad-empty, Ad-flag-SHP WT or R57W (0.5-1.0x10^9 active viral particles in 200 □l PBS). Seven days after infection, mice were fasted for 6 h and i.p. injected with glucose solution (Sigma, Inc, 2g/kg) or insulin (Sigma, Inc, 2units/kg) and glucose levels were measured using an Accu-chek Aviva glucometer (Roche, Inc).

q-RTPCR

Total RNA was isolated using Trizol reagent (Invitrogen), cDNA was synthesized using a reverse transcriptase kit (Promega), and q-RTPCR was performed with an icycler iQ (Biorad). The amount of mRNA for each gene was normalized to that of 36B4 mRNA. Primer sequences are shown in Supplemental Information.

Mass spectrometry analyses

Flag-human SHP was expressed in HepG2 cells (three 15-cm plates per group) by adenoviral infection and 48 h later, cells were treated with 5 μM MG132 for 4 h to inhibit degradation and then further treated with CDCA for 1 h. Flag-SHP was isolated in RIPA (SDS) lysis buffer using M2 agarose and then incubated with purified PRMT5 (purchased from Abnova) and unlabeled SAM at 30^{o}C for 1 h. Proteins were separated by SDS-PAGE, visualized with colloidal staining and flag-SHP bands were excised, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. To identify SHP interacting proteins *in vivo*, mice were infected with Ad-flag-human SHP and 5 days later, mice were fed normal chow or CA chow for 3 h and liver extracts were prepared. The flag-SHP complex was isolated in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA and 0.1% NP40) using M2 agarose and interacting proteins were identified using LC-MS/MS.

In vitro and in cell methylation assays

HepG2 cells (15-cm plate/group) infected with Ad-flag-SHP were treated with MG132 for 4 h and further treated with CDCA or vehicle for 1 h. Flag-SHP was isolated using M2 agarose and then incubated with purified PRMT5 and radioactively labeled or unlabeled SAM in methylation buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM DTT) at 30^{o}C for 1 h as previously

described (9). Proteins were separated by SDS-PAGE and methylated SHP was detected by autoradiography or western analysis, respectively. For in vitro assays, GST-SHP was incubated with purified PRMT5 and SAM in methylation buffer at 30^{o}C for 1 hr.

GST pull down and CoIP assays

Standard GST pull down assays and CoIP were performed as described previously (9, 10, 25). Briefly for CoIP assays, cells were transfected with expression plasmids or infected with adenoviral vectors and treated with vehicle or CDCA for 1- 3 hr. Cell extracts were prepared in CoIP buffer (50 mM Tris, pH. 8.0, 150 mM NaCl, 2 mM EDTA, 0.3% NP40, 10% glycerol) supplemented with protease inhibitors, DTT, and phosphatase inhibitors (Na orthovanadate, sodium fluoride, sodium pyrophosphate, sodium molybdate). Cell pellets were briefly sonicated and centrifuged. Supernatant was incubated with 1-2 □g antibodies for 30 min and 30 μl of 25% protein G agarose were added. Two h later, samples were washed with the CoIP buffer for 3 times and proteins were separated by SDS-PAGE and detected by western analysis.

In vivo chromatin IP (ChIP) and re-ChIP assays

ChIP assays in mouse liver were carried out essentially as described (9, 10, 18, 24, 25). Re-ChIP assays were performed as described previously (10). Briefly, chromatin precipitated by M2 antibody was extensively washed, eluted by adding 50 □l of 10 mM DTT at 37 ^{o}C for 30 min and then, diluted (20-fold) with buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), re-precipitated using antibodies to SHP and its interacting proteins. Occupancy of proteins at the target gene promoters was examined using semi-quantitative PCR. Primer sequences are shown in Supplemental Information.

Results

PRMT5 interacts with SHP in response to bile acid signaling

The association of mutations in Arg residue of SHP with the metabolic syndrome in humans (7, 8, 27) (Fig. S2.1) led us to examine whether PTMs at Arg might be important for regulating SHP activity. To identify enzymes that catalyze PTMs and interact with SHP, human flag-SHP was expressed in mouse liver by infection with an adenoviral expression vector, flag-SHP was affinity purified, and associated proteins were identified by mass spectrometric analysis (Fig. 2.1A). PRMT5 was associated with SHP in mice fed a primary bile acid, cholic acid (CA) (Fig. 2.1B). To confirm this result, endogenous SHP was immunoprecipitated from liver nuclear extracts and PRMT5 in the anti-SHP immunoprecipitates was detected by western analysis. Interaction of SHP with PRMT5 was dramatically increased in mice fed CA (Fig. 2.1C). Similar results were observed in HepG2 cells treated with a primary bile acid, chenodeoxycholic acid (CDCA) (Fig. S2.2). To test whether PRMT5 directly interacts with SHP, in vitro GST pull down assays were performed (Fig. 2.1D-F). PRMT5 directly interacted with N-terminal and C-terminal fragments as well as full length SHP, indicating that two independent PRMT5 binding domains were present in SHP (Fig. 2.1F). Similar results were obtained with GST pull down assays using ^{35}S-labeled PRMT5 (Fig. S2.3). These results show that PRMT5 interacts with SHP in mouse liver in vivo in response to bile acid signaling.

PRMT5 augments SHP repression activity

To test whether PRMT5 interaction with SHP is functionally relevant, cell-based reporter assays were performed using gain- or loss-of-function experiments. In a Gal4 reporter system, overexpression of SHP inhibited the transactivation mediated by Gal4-HNF-4 (Fig. 2.2A, lanes 2-3) and Gal4-LRH-1 (Fig. S2.4). Exogenous expression of PRMT5 augmented SHP-mediated

inhibition of HNF-4α/PGC1α (Fig. 2.2A, lanes, 3-5) and LRH-1 (Fig. S2.4). Conversely, depletion of endogenous PRMT5 by siRNA or overexpression of catalytically inactive PRMT5 mutant reversed SHP inhibition of HNF-4α/PGC1α (Fig. 2.2A, B). Importantly, the enhancement of SHP repression by PRMT5 was not observed when SHP was downregulated by siRNA (Fig. 2.2C). These results, together with CoIP studies (Fig. 2.1), suggest that PRMT5 enhances repression of HNF-4α/PGC1α and LRH-1 transactivation probably through its interaction with SHP.

Effects of hepatic PRMT5 depletion on expression of SHP metabolic target genes

To determine the functional role of PRMT5 in metabolic regulation by SHP, endogenous PRMT5 in HepG2 cells was down regulated and expression of known SHP metabolic target genes was examined. CDCA treatment resulted in decreased mRNA levels of the bile acid biosynthetic genes, CYP7A1 and CYP8B1, lipogenic genes, FAS and SREBP-1c, and the gluconeogenic genes, glucose-6-phosphatase and PEPCK (Fig. 2.2D). Downregulation of PRMT5 reversed these effects on expression of the metabolic genes, except that of PEPCK (Fig. 2.2D). These results indicate that PRMT5 plays a role in the regulation of lipid and glucose metabolism by SHP.

To explore the in vivo significance of PRMT5 in metabolic regulation by SHP, endogenous PRMT5 in mouse liver was depleted by adenoviral vector-mediated expression of siRNA and expression of known SHP metabolic target genes was examined (Fig. 2.2E). Hepatic PRMT5 protein levels were markedly decreased, whereas control lamin levels were not changed (Fig. 2.2F). Depletion of PRMT5 resulted in increased mRNA levels of the bile acid biosynthetic genes, Cyp7a1 and Cyp8b1, lipogenic genes, Fas and Srebp-1c, and the gluconeogenic gene, Glucose-6-phosphatase, but not the PEPCK gene (Fig. 2.2G). Consistent

with these results, bile acid pools from liver, gall bladder, and intestine, and liver triglyceride levels were significantly increased in these mice (Fig. 2.2H, I). These results demonstrate that PRMT5 plays a role in the regulation of liver metabolism by SHP.

PRMT5 methylates SHP in vitro and in vivo

To test if PRMT5 can methylate SHP, GST-SHP or control GST was incubated with purified PRMT5 and ^{3}H-S-adenosyl methionine (SAM) in vitro. GST-SHP was methylated by PRMT5 in the presence of ^{3}H-SAM (Fig. 2.3A, lane 3). Similar results were observed with unlabeled SAM and detection by western analysis using antisera to methylated Arg (Fig. S2.5). To directly test whether endogenous SHP in mouse liver is a target of post-translational methylation by PRMT5, endogenous PRMT5 in mouse liver was depleted using adenoviral siRNA as described before (Fig. 2.2D) and then, methylation of endogenous SHP was detected by immunoprecipitation under stringent condition with SDS-containing buffer followed by western analysis (Fig. 2.3B, top). Arg-methylated SHP levels were markedly decreased in PRMT5-depleted liver compared to control mice (Fig. 2.3B, bottom).

PRMT5 methylates SHP at Arg-57 after CDCA treatment

In order to determine the functional roles of post-translational methylation of SHP, Arg residue(s) methylated by PRMT5 were identified using tandem mass spectrometry (MS/MS) (Fig. 2.3C). Methylated SHP was dramatically increased by CDCA treatment of HepG2 cells (Fig, 2.3D, lane 4). Only methylation at Arg-57 was detected in purified SHP after CDCA treatment (Fig. 2.3E, F). Arg-57 is highly conserved in mammals (Fig. S2.6) and intriguingly, a natural mutation, R57W is associated with the metabolic syndrome in humans (Fig. S2.1) (7, 8, 27). Mutation of

Arg-57 abolished the methylation of SHP (Fig. 2.3G, H), confirming that Arg-57 is the major site methylated by PRMT5. These proteomic and biochemical studies demonstrate that PRMT5 methylates SHP at Arg-57 and suggest that bile acid signaling increases methylation.

Arg-57 methylation augments SHP repression function

To test the functional relevance of Arg-57 methylation, the activity of the R57W SHP mutant was examined by cell-based reporter assays. Enhancement of SHP repression of HNF-4 (Fig. 2.4A) and LRH1 (Fig. 2.4B) by the R57W mutant was substantially less when compared to the wild type (WT) protein. Repression effects of SHP were markedly reduced, although not completely, by a more conservative R57K mutation (Fig. 2.4C). The continued, but markedly decreased, effects of the R57K mutant suggest that methylation enhances SHP activity, but is not absolutely required for its activity. Further these data strengthen the conclusion that decreased methylation of R57, rather than nonspecific conformational changes, largely contributes to decreased SHP activity. Comparable expression levels of WT SHP and the mutant proteins were detected, although mobility of the R57K mutant was slightly altered (Fig. 2.4B, inset). Importantly, enhancement of SHP repression by PRMT5 was not observed with the R57W and R57K mutants (Fig. 2.4D). These results suggest that methylation at Arg-57 by PRMT5 augments SHP repression function.

Arg-57 SHP mutant shows impaired interaction with its known cofactors

To identify molecular mechanisms by which Arg-57 methylation augments SHP repression activity, we first tested whether methylation might stabilize SHP. The half-life of the R57W mutant, however, was similar to the WT SHP and if anything, the stability of the R57W mutant

increased since its steady state levels were increased compared to WT protein (Fig. 2.5A). The decreased SHP activity of R57W, thus, cannot be explained by reduced protein stability.

Next, we examined whether methylation of SHP increases interaction with its known chromatin modifying repressive cofactors, mSin3A, HDAC1, G9a, and Brm (9, 16, 25). Interaction with a well known SHP interacting DNA binding factor, LRH-1 was also examined. Flag-SHP was isolated from untreated or CDCA-treated HepG2 cells and incubated in vitro with PRMT5. Treatment of cells with CDCA resulted in increased methylation of SHP and interaction with its cofactors (Fig. 2.5B, lane 3) and substantially increased the in vitro methylation of SHP by PRMT5 (Fig. 2.5B, lane 4). The increased methylation correlated with increased interactions of SHP with Brm and HDAC1, but not with G9a and LRH-1 (Fig. 2.5B). These results suggest that increased methylation of SHP by CDCA treatment selectively increases its interaction with cofactors.

To further test if R57 methylation is important for increased interaction between SHP and its cofactors, we performed CoIP studies using the R57K and R57W mutants. HepG2 cells were transfected with expression plasmids for flag-SHP and its cofactors, and the interaction between SHP and these cofactors was examined. CDCA treatment dramatically increased methylation of SHP WT and interaction with mSin3A, HDAC1, Brm, PRMT5, and G9a (Fig. 2.5C). In contrast, these increased interactions were not observed with R57W and largely decreased with the R57K mutant. Consistent with in vitro CoIP studies (Fig. 2.5B), decreased SHP interaction with G9a was not observed with R57K and R57W (Fig. 2.5C and Fig. S2.7), suggesting that G9a is present in a SHP complex in hepatic cells and this interaction is independent of methylation at Arg-57.

These data demonstrate that methylation of SHP is important for enhanced interaction with some, but not all, of its cofactors (Fig. 2.5D).

Occupancy of PRMT5 and SHP at the Cyp7a1 promoter in vivo is increased after bile acid treatment

To test whether PRMT5 occupancy at the Cyp7a1, a well known SHP target (4, 12, 22, 30), is increased after bile acid treatment in mouse liver and whether the Cyp7a1 promoter is co-occupied by SHP and PRMT5, we performed re-chromatin IP (re-ChIP) assays. Chromatin was immunoprecipitated first with SHP antisera and then, eluted chromatin was re-precipitated with antisera to PRMT5 and other known SHP interacting cofactors. Occupancy of SHP, PRMT5, G9a, and Brm at the promoter was increased by CA feeding, while that of the transcriptional activity marker RNA polymerase II was decreased (Fig. 2.6A). Occupancy of PRMT5 at the human CYP7A1 gene promoter was also increased after CDCA treatment of HepG2 cells (Fig. 2.6B). These results suggest that PRMT5 as well as G9a, Brm, and SHP are recruited to the Cyp7a1 promoter after bile acid treatment in vivo, resulting in gene repression.

Methylation-defective R57W mutant shows impaired recruitment of its cofactors to metabolic target genes

Using re-ChIP assays in mouse livers expressing flag-SHP WT or the R57W mutant, we next examined the effect of the R57W mutation on recruitment of SHP cofactors to the promoters of three well known metabolic target genes, Cyp7a1, Cyp8b1, and Srebp-1c (9, 16, 25, 31, 38). At each promoter, similar occupancy of flag-SHP or R57W was detected, which is consistent with similar interaction of both to the DNA binding protein LRH-1 (Fig. 2.5B). Occupancy of

PRMT5 and Brm was markedly decreased with the R57W mutant for all three genes (Fig. 2.6C). Consistent with the CoIP studies (Fig. 2.5C), occupancy of G9a at these promoters was not decreased in mice expressing the R57W mutant (Fig. 2.6C), suggesting that Arg-57 methylation is not required for G9a recruitment. These in vivo re-ChIP studies, together with CoIP studies (Fig. 2.5C), suggest that methylation of Arg-57 is important for interaction of SHP with HDAC1 and Brm, but not with G9a, and recruitment of these cofactors to SHP target gene promoters.

Hepatic overexpression of the R57W mutant reverses repression of SHP metabolic targets in a gene-selective manner

To determine the physiological significance of Arg-57 methylation in metabolic regulation, the effects of the methylation-defective R57W mutant on expression of SHP target metabolic genes was examined in vivo (Fig. 2.7A). As in the cell culture studies (Fig. 2.3H), methylation of SHP was severely impaired in mice expressing the R57W mutant compared to WT (Fig. 2.7B, C). Hepatic expression of SHP WT led to decreased expression of bile acid biosynthetic genes, Cyp7a1 and Cyp8b1, lipogeneic genes, Fas and Srebp-1c, and bile acid transporter genes, Bsep and Ntcp (Fig. 2.7D) as previously reported (1, 3). Exogenous expression of SHP WT also decreased expression of the gluconeogenic genes, Pepck and G-6-pase, but effects were not statistically significant. Interestingly, mutation of Arg-57 reversed the effects in some target genes but not others like Cyp7a1 (Fig. 2.7D, Fig. S2.8), suggesting that Arg-57 methylation affects SHP function in a gene-specific manner. Consistent with gene expression studies, liver triglyceride levels and total bile acid pool were decreased in mice exogenously expressing WT protein, but substantially elevated in mice expressing the R57W mutant (Fig. 2.7E, F). In contrast, glucose and insulin tolerance were similarly increased in mice overexpressing either

SHP WT or the R57W mutant (Fig. 2.7G and Fig. S2.9). These in vivo studies demonstrate a novel function of PRMT5 as a critical regulator of SHP in metabolic function and further suggest that R57 methylation by PRMT5 may contribute to gene-specific and perhaps metabolic pathway-specific repression, possibly by differential interaction with and recruitment of known SHP's chromatin modifying repressive cofactors (Fig.s 2.5 and 2.6).

Discussion

Our studies have identified PRMT5 as an important in vivo regulator of SHP in metabolic function. First, proteomic and CoIP studies revealed that the interaction of PRMT5 with SHP was dramatically increased in liver in response to bile acid signaling. Second, tandem mass spectrometry and biochemical studies have shown that methylation of SHP at Arg-57 by PRMT5 was substantially increased after bile acid treatment. Third, re-ChIP and CoIP studies have revealed that mutation of Arg-57 led to selectively decreased interaction of SHP with Brm, mSin3A, and HDAC1, but not with G9a, and subsequent recruitment of these cofactors to SHP's target genes. Finally, functional *in vivo* experiments have shown that hepatic overexpression of methylation-defective R57W or depletion of PRMT5 both reversed the repression of SHP metabolic target genes in a gene-selective manner. Consistent with gene expression studies, the inhibitory effects of SHP WT on bile acid pool and liver triglyceride levels were impaired with the mutation of Arg-57, but interestingly, the effects on glucose and insulin tolerance were not altered.

Naturally-occurring heterozygous mutations, including R57W, in the SHP gene have been reported in humans with type II diabetes, obesity, and fatty liver (7, 8, 27) confirming the important metabolic functions of SHP. The effects of the R57W mutation on gene expression and triglyceride and bile acid levels in mice are consistent with its association with human

metabolic disease. Hepatic expression of the R57W mutant markedly increased lipogenic and bile acid synthetic gene expression in comparison to expression of wild type SHP. These changes in gene expression resulted in elevated hepatic triglyceride levels and the total bile acid pool. Similar effects were observed with the depletion of PRMT5, which further strengthens the conclusion that PRMT5 enhances SHP repression function by methylation of Arg-57. In addition, conformational changes in R57W may contribute to the reduced activity of SHP since the more conservative R57K mutation resulted in smaller effects on SHP activity. Taken together, these results provide a possible explanation of why the R57W mutation is associated with metabolic syndrome in humans.

Understanding how transcription factors regulate their target genes in a gene-specific manner has been a long-standing question. PTMs, including methylation, may provide distinct protein interacting interfaces that allow differential interaction with transcriptional cofactors and may contribute to gene-specific regulation (15, 17, 21). Previous studies have shown that post-translational methylation of p53 by PRMT5 is important for determining whether cells enter cell cycle arrest or apoptosis by repressing different sets of target genes (15). In this study, we have found that mutation of Arg-57 reversed the suppression of some, but not all, metabolic genes by SHP in mouse liver. Such gene-specific effects may be partly due to differential interaction of methylated SHP with its cofactors as observed from CoIP and re-ChIP studies. For example, regulation of genes specifically dependent on the cofactor G9a, such as Cyp7a1 (9), might be independent of Arg-57 methylation since the mutation does not reduce levels of G9a in the SHP complex. In contrast, regulation of genes more dependent on the cofactors, Brm and HDAC1, such as Cyp8b1 and Srebp1-c genes, would be affected by methylation since mutation of Arg-57 reduces the interaction of SHP with these cofactors. Similar effects were observed with both the

R57W mutant of SHP (Fig. 2.7D) and the downregulation of PRMT5 (Fig. 2.2F), which provides strong evidence that PRMT5-catalyzed Arg methylation enhances SHP repression of metabolic genes. An exception was the effects on Cyp7a1 for which the R57W was similar to wild type SHP (Fig. 2.7D), while downregulation of PRMT5 increased Cyp7a1 expression (Fig. 2.2F). PRMT5 may regulate Cyp7a1 by other indirect mechanisms in addition to methylation of Arg-57 in SHP, such as histone methylation at the target genes.

The activity of most nuclear receptors is regulated by ligand binding (23), but SHP was discovered as an orphan receptor (32) and its endogenous ligand is not known. In this regard, modulation of SHP activity by PTMs in response to physiological stimuli would be an effective alternative way to control its activity and/or stability. SHP is a well known component of cellular sensor systems for bile acid signaling (1, 3). Bile acids serve not only dietary roles in the absorption of fat-soluble nutrients but also function as endocrine signaling molecules that trigger genomic and non-genomic signaling pathways (4, 13, 30, 33). We recently reported that bile acid signaling activates ERK, which phosphorylates SHP at Ser-26, which increases SHP stability in hepatocytes (26). Thus, in addition to SHP gene induction by the bile acid-activated nuclear receptor FXR (12, 22), modulation of SHP stability and repression activity by PTMs are likely to be important in the mediation of bile acid signaling by SHP. To our knowledge, this study is the first demonstration that SHP repression activity is increased by posttranslational modification in response to bile acid signaling.

Since this study demonstrates increased methylation of SHP in response to elevated bile acid levels, it will be important to determine whether specific kinase(s) in bile acid signaling pathways are involved in Arg methylation by PRMT5 and whether methylation of SHP affects or is affected by other PTMs. FGF15/19 signaling is activated in response to elevated bile acid

levels in the enterohepatic system in vivo (14), so it will be also important to determine whether FGF15/19 signaling enhances SHP activity by Arg methylation by PRMT5. Furthermore, it will be interesting to determine whether decreased methylation of SHP is associated with metabolic disease, which is analogous to our recent findings that acetylation of FXR is normally dynamically regulated by p300 acetylase and SIRT1 deacetylase but highly elevated in metabolic disease states (17, 18).

SHP plays an important role in controlling lipid and glucose levels by inhibiting metabolic target genes in the liver and other metabolic tissues and is also involved in cell proliferation, apoptosis, and reproduction (1, 3, 11, 35, 36, 39). Given that SHP plays important roles in such diverse mammalian physiology, PTMs may provide a mechanism of selective regulation of genes in biological processes. Further, targeting post-translational modifications of SHP may be an effective therapeutic strategy by controlling selected groups of genes to treat SHP-related human diseases, such as metabolic syndrome, cancer, and infertility.

Acknowledgements

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Figures and legends

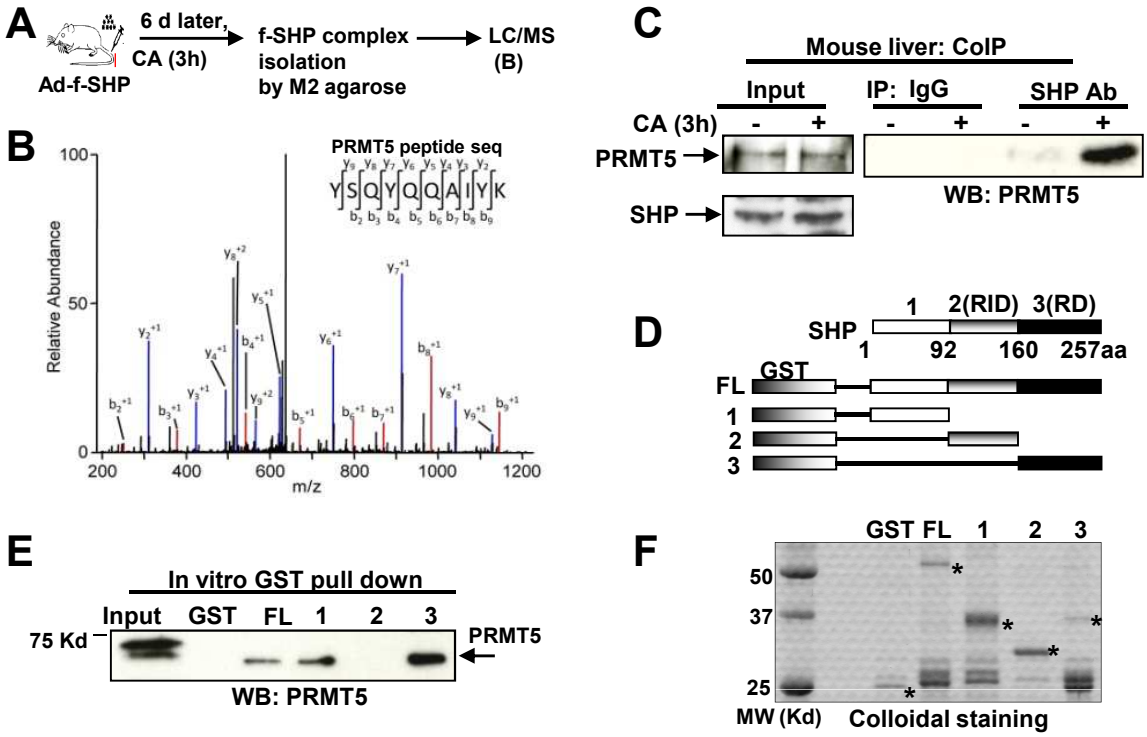


Fig. 2.1. PRMT5 interacts with SHP after bile acid treatment and augments SHP activity. (A) Mice were injected via tail veins with adenoviral vector expressing flag-SHP, and 6 days later, mice were fed 0.5% CA-supplemented chow for 3 h and liver extracts were prepared. The flag-SHP complex was isolated using M2 agarose, and interacting proteins were identified by tandem LC-MS/MS. (B) Tandem MS spectrum of a PRMT5 peptide identified in the SHP complex. (C) Mice were fed normal or CA chow for 3 h and the interaction of endogenous SHP with PRMT5 in liver extracts was examined by CoIP. (D) Schematic diagrams of the receptor interacting domain (RID) and intrinsic repression domain (RID) in SHP are shown.(E) Amounts of GST or GST-SHP full length (FL) or deletion mutants used in the reactions were visualized by staining. GST or GST-SHP proteins are indicated by asterisks. (F) Interaction of PRMT5 with GST-SHP proteins was detected by western analysis using PRMT5 antibody.

Fig. 2.2

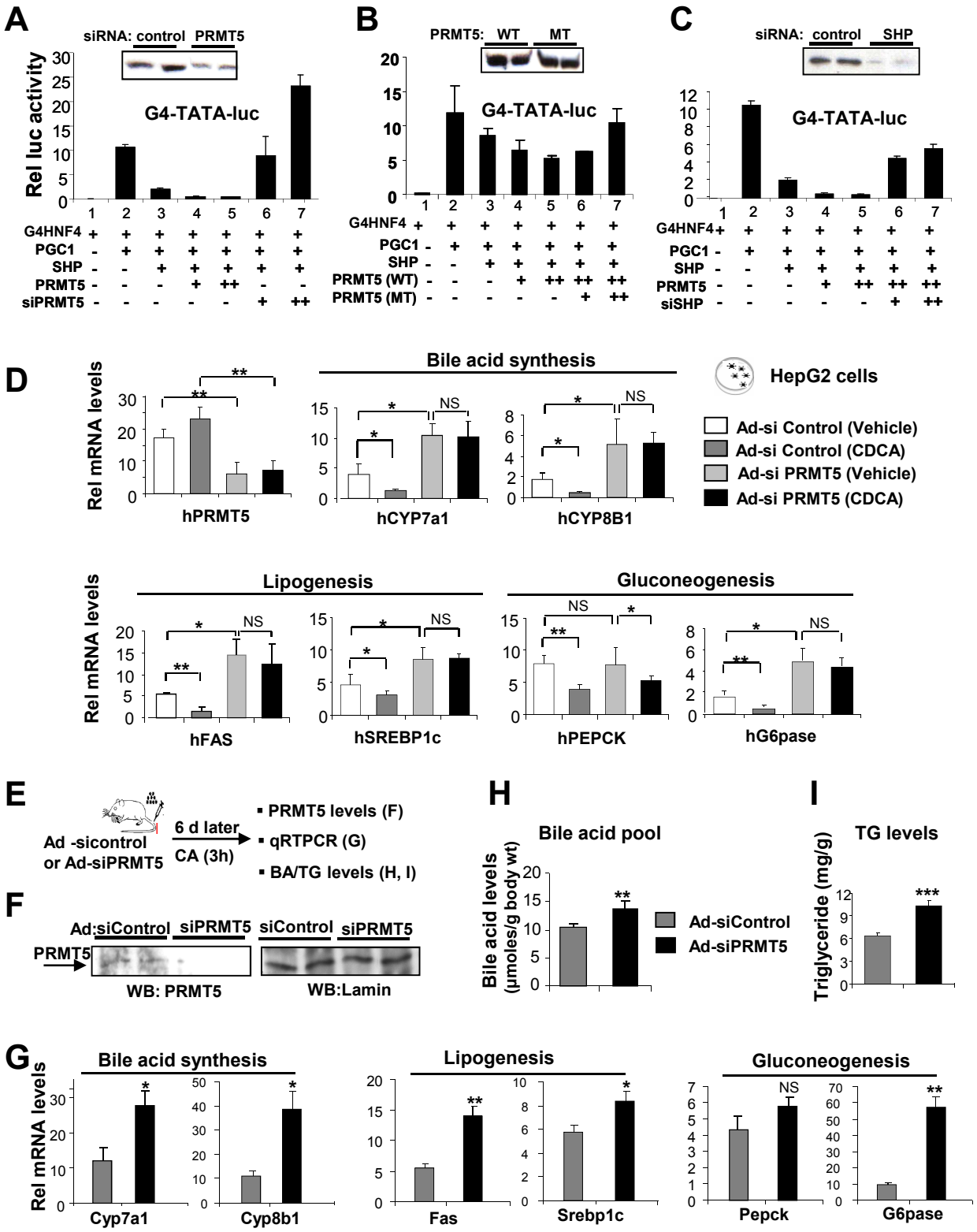

A
siRNA: control PRMT5
Rel luc activity
G4-TATA-luc
G4HNF4
PGC1
SHP
PRMT5
siPRMT5
B
PRMT5: WT MT
G4-TATA-luc
G4HNF4
PGC1
SHP
PRMT5 (WT)
PRMT5 (MT)
C
siRNA: control SHP
G4-TATA-luc
G4HNF4
PGC1
SHP
PRMT5
siSHP
D
Rel mRNA levels
Bile acid synthesis
HepG2 cells
Ad-si Control (Vehicle)
Ad-si Control (CDCA)
Ad-si PRMT5 (Vehicle)
Ad-si PRMT5 (CDCA)
hPRMT5
hCYP7a1
hCYP8B1
Lipogenesis
Gluconeogenesis
hFAS
hSREBP1c
hPEPCK
hG6pase
NS
E
Ad -sicontrol
or Ad-siPRMT5
6 d later
CA (3h)
▪ PRMT5 levels (F)
▪ qRTPCR (G)
▪ BA/TG levels (H, I)
F
Ad:siControl siPRMT5 siControl siPRMT5
PRMT5
WB: PRMT5
WB:Lamin
H
Bile acid pool
Bile acid levels (µmoles/g body wt)
Ad-siControl
Ad-siPRMT5
I
TG levels
Triglyceride (mg/g)
G
Bile acid synthesis
Rel mRNA levels
Cyp7a1
Cyp8b1
Lipogenesis
Fas
Srebp1c
Gluconeogenesis
Pepck
G6pase

Fig. 2.2. PRMT5 augments repression activity by SHP. (A-C) HepG2 cells were transfected with a Gal4-TATA-luc reporter and expression plasmids as indicated and 36 hr later, cells were treated with CDCA overnight and reporter assays were performed. The values for firefly luciferase activities were normalized by dividing by the b-galactosidase activities. The mean and SEM, n=3, are plotted. (D) HepG2 cells were infected with Ad-siPRMT5 or control Ad-siRNA and then 2 days later, cells were treated with vehicle or 50 mM CDCA overnight and mRNA levels of bile acid synthetic, lipogenic, and gluconeogenic genes were measured by q-RTPCR. (E-I) Effects of hepatic PRMT5 depletion on expression of known SHP target genes and metabolic outcomes. (E) Experimental outline for in vivo PRMT5 depletion experiments. (F) Endogenous PRMT5 levels were detected by western analysis. (G) Expression of SHP target genes was examined. (H, I) Bile acid pool and hepatic triglyceride levels were measured. (G-I) The mean and SEM (n=3) are plotted. Statistical significance was determined using the Student's t test. *, **, and NS indicate $p<0.05$, $p<0.01$, and statistically not significant, respectively.

Fig. 2.3

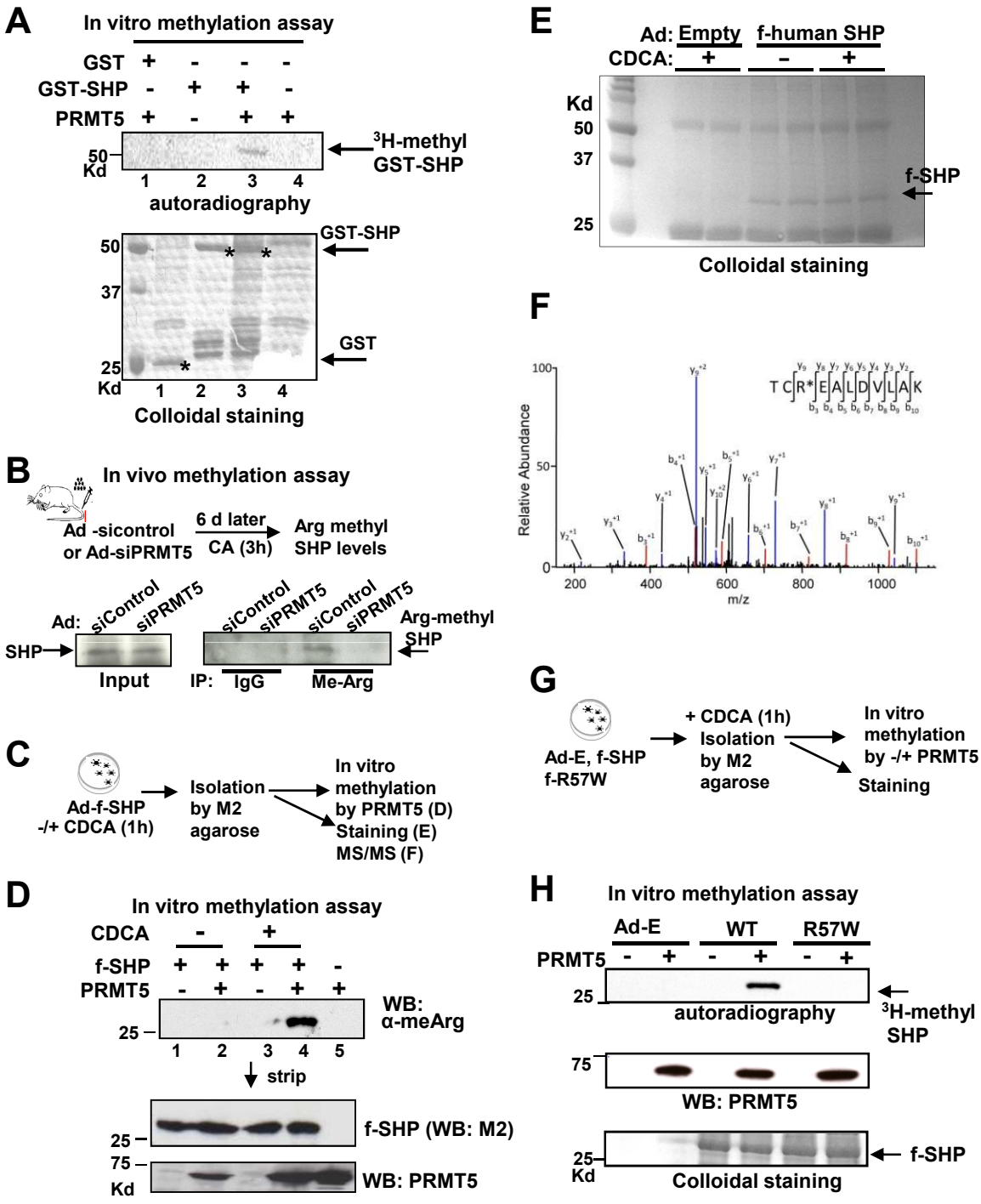

A
In vitro methylation assay
GST + - - -
GST-SHP - + + -
PRMT5 + - + +
50
Kd
1 2 3 4
^{3}H-methyl GST-SHP
autoradiography
50
37
25
Kd
1 2 3 4
GST-SHP
GST
Colloidal staining
B
In vivo methylation assay
Ad -sicontrol or Ad-siPRMT5
6 d later
CA (3h)
Arg methyl SHP levels
Ad:
siControl
siPRMT5
siControl
siPRMT5
siControl
siPRMT5
Arg-methyl SHP
SHP
Input
IP:
IgG
Me-Arg
C
Ad-f-SHP
-/+ CDCA (1h)
Isolation by M2 agarose
In vitro methylation by PRMT5 (D)
Staining (E)
MS/MS (F)
D
In vitro methylation assay
CDCA - +
f-SHP + + + + -
PRMT5 - + - + +
25
1 2 3 4 5
WB: α-meArg
strip
25
f-SHP (WB: M2)
75
25
Kd
WB: PRMT5
E
Ad: Empty f-human SHP
CDCA: + – +
Kd
50
37
25
f-SHP
Colloidal staining
F
100
50
0
Relative Abundance
200
400
600
800
1000
m/z
T C R* E A L D V L A K
G
Ad-E, f-SHP
f-R57W
+ CDCA (1h)
Isolation by M2 agarose
In vitro methylation by -/+ PRMT5
Staining
H
In vitro methylation assay
Ad-E
WT
R57W
PRMT5 - + - + - +
25
autoradiography
^{3}H-methyl SHP
75
WB: PRMT5
25
Kd
f-SHP
Colloidal staining

Fig. 2.3. PRMT5 methylates SHP at Arg-57 after bile acid treatment. (A) GST-SHP or GST was incubated with purified PRMT5 and 3H-S-adenosyl methionine and methylated SHP was detected by autoradiography (top). Similar GST-SHP amounts were used in the reaction (bottom). (B) Experimental outline for in vivo SHP methylation assays (top). Hepatic PRMT5 was down regulated by adenovirally expressed siRNA for PRMT5, and endogenous SHP was immunoprecipitated under stringent condition using SDS-containing buffers. Arg-methylated SHP was detected by western analysis (bottom). (C) Experimental outline for MS/MS analysis. Flag-human SHP was isolated from HepG2 cells treated with vehicle or CDCA for 1 h and incubated with PRMT5 and SAM. (D) Methylated SHP was detected by western analysis. The membrane was stripped and flag-SHP and PRMT5 levels were detected. (E) After *in vitro* methylation, proteins were separated by PAGE and visualized by colloidal staining. Flag-SHP bands (arrow) were excised for LC-MS/MS analysis. (F) The MS/MS spectrum of the SHP peptide containing methylated Arg-57. (G) Experimental outline: HepG2 cells infected with Ad-flag-SHP WT or the Ad-flag-R57W were treated with CDCA for 1 h, and flag-SHP was isolated for *in vitro* assays. (H) ^{3}H-methylated SHP was detected by autoradiography (top) and PRMT5 (middle) and f-SHP levels (bottom) by western analysis and colloidal staining, respectively.

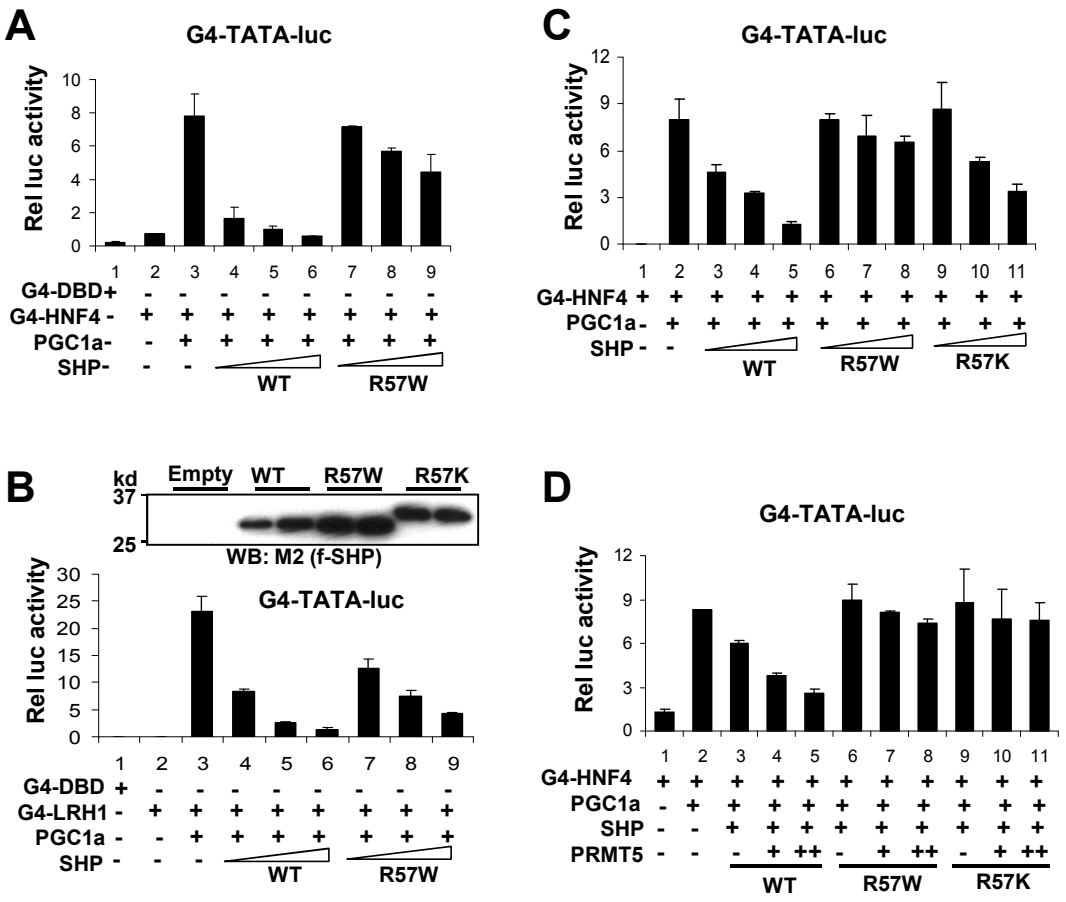


Fig. 2.4. Arg-57 methylation is important for SHP repression activity. (A-D) HepG2 cells transfected with plasmids as indicated (for plasmid amounts, see Materials and Methods) were treated with CDCA overnight and reporter assays were performed. The triangles represent increasing amounts of the flag-SHP vectors. The values for firefly luciferase activities were normalized by dividing with the b-galactosidase activities. The mean and SEM is plotted (n=3). In B, expression levels of flag-SHP wild type (WT), R57W, and R57K from duplicate samples are shown at the top.

Fig. 2.5

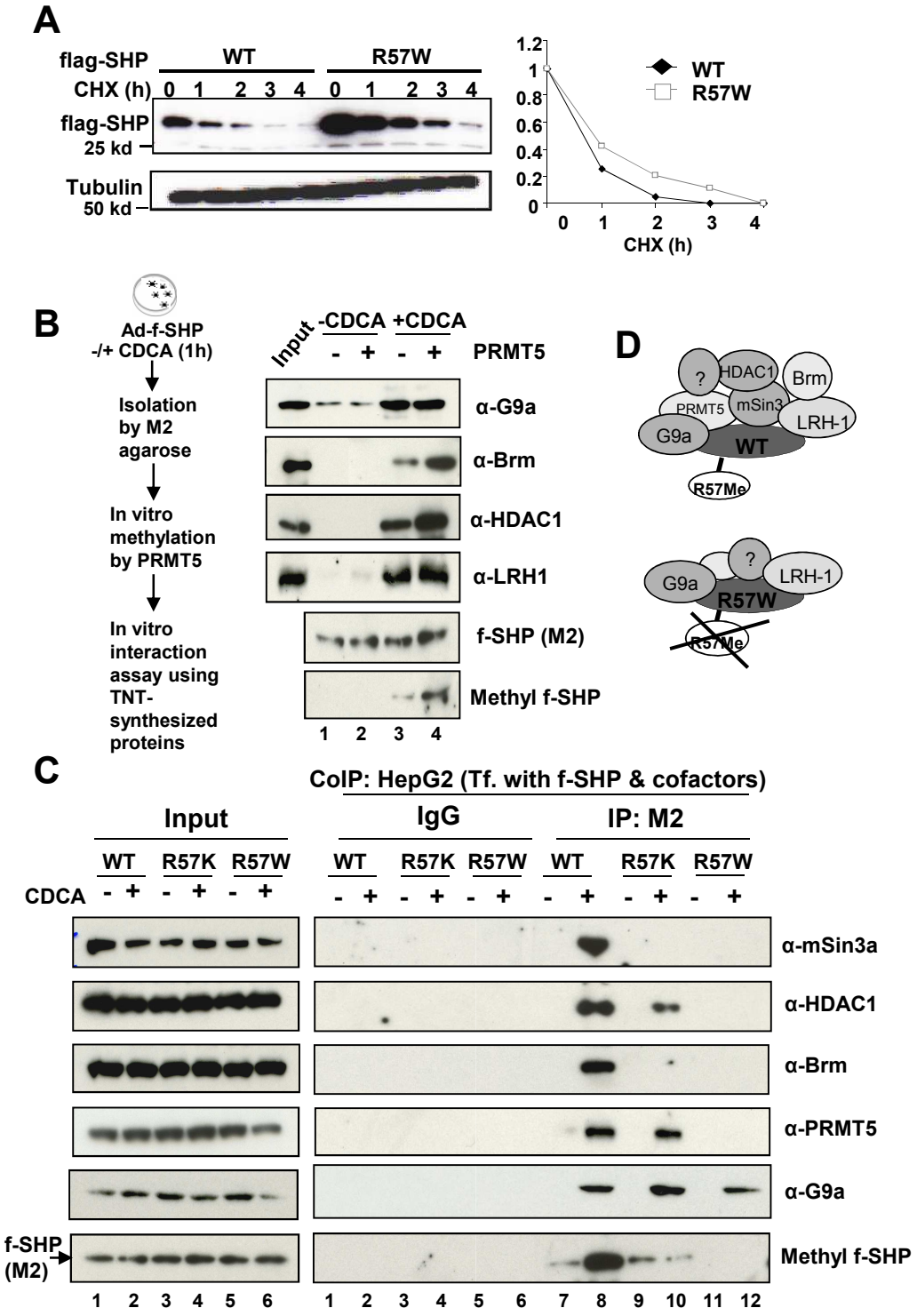

A
flag-SHP
WT
R57W
CHX (h) 0 1 2 3 4 0 1 2 3 4
flag-SHP
25 kd
Tubulin
50 kd
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4
CHX (h)
WT
R57W
B
Ad-f-SHP
-/+ CDCA (1h)
Isolation by M2 agarose
In vitro methylation by PRMT5
In vitro interaction assay using TNT-synthesized proteins
Input
-CDCA +CDCA
- + - +
PRMT5
α-G9a
α-Brm
α-HDAC1
α-LRH1
f-SHP (M2)
Methyl f-SHP
1 2 3 4
D
?
HDAC1
Brm
PRMT5
mSin3
LRH-1
G9a
WT
R57Me
?
G9a
LRH-1
R57W
R57Me
C
CoIP: HepG2 (Tf. with f-SHP & cofactors)
Input
IgG
IP: M2
WT R57K R57W WT R57K R57W WT R57K R57W
CDCA - + - + - + - + - + - + - + - + - +
α-mSin3a
α-HDAC1
α-Brm
α-PRMT5
α-G9a
f-SHP (M2)
Methyl f-SHP
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2.5. Mutation of R57 in SHP does not affect stability but selectively impairs interaction with its known chromatin modifying cofactors. (A) HepG2 cells infected with Ad-flag-SHP WT or R57W were treated with cycloheximide (CHX) (10 mg/ml) and flag-SHP levels were detected by western analysis. Band intensities were measured by densitometry and the intensities relative to the 0 min time point were plotted (right panel). (B) Experimental outline (left). Flag-SHP was isolated by affinity binding to M2 agarose and incubated with the indicated proteins synthesized from the transcription and translation (TNT) system. Flag-SHP was immunoprecipitated and SHP-interacting proteins and methylated SHP were detected by western analysis (right). (C) HepG2 cells were cotransfected with expression plasmids for flag-SHP WT or mutants as indicated. Proteins were immunoprecipated with M2 antibody for Flag or IgG control and proteins in the immunoprecipitates were detected by western analysis using each of the indicated antibodies or SYM10 for methylated SHP. (D) A schematic diagram of transcription regulators interacting with flag-SHP WT (top) or the R57W mutant (bottom) is shown.

Fig. 2.6

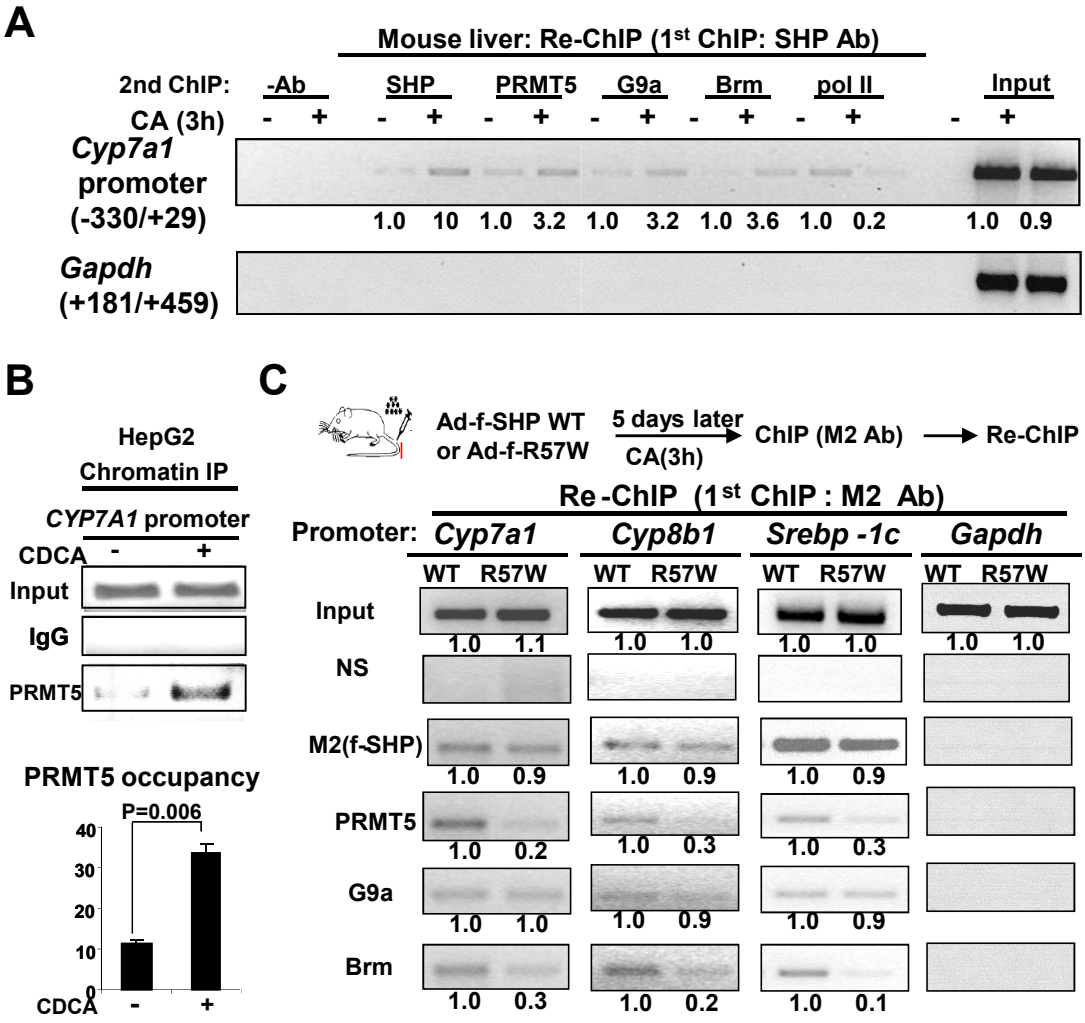

A
Mouse liver: Re-ChIP (1st ChIP: SHP Ab)
2nd ChIP: -Ab SHP PRMT5 G9a Brm pol II Input
CA (3h) - + - + - + - + - + - + - +
Cyp7a1 promoter (-330/+29)
1.0 10 1.0 3.2 1.0 3.2 1.0 3.6 1.0 0.2 1.0 0.9
Gapdh (+181/+459)
B
HepG2
Chromatin IP
CYP7A1 promoter
CDCA - +
Input
IgG
PRMT5
PRMT5 occupancy
P=0.006
40
30
20
10
0
CDCA - +
C
Ad-f-SHP WT or Ad-f-R57W
5 days later
CA(3h)
ChIP (M2 Ab)
Re-ChIP
Re-ChIP (1st ChIP : M2 Ab)
Promoter: Cyp7a1 Cyp8b1 Srebp -1c Gapdh
WT R57W WT R57W WT R57W WT R57W
Input
1.0 1.1 1.0 1.0 1.0 1.0 1.0 1.0
NS
M2(f-SHP)
1.0 0.9 1.0 0.9 1.0 0.9
PRMT5
1.0 0.2 1.0 0.3 1.0 0.3
G9a
1.0 1.0 1.0 0.9 1.0 0.9
Brm
1.0 0.3 1.0 0.2 1.0 0.1

Fig. 2.6. Mutation of R57 in SHP impairs recruitment of Brm and PRMT5, but not G9a, to metabolic target genes. (A) Mice were fed normal or CA chow and re-ChIP assays were performed. Chromatin was immunoprecipitated with SHP antibody first, eluted, and then re-precipitated with a second antibody as indicated. Semi-quantitative PCR was performed to detect occupancy at the Cyp7a1 promoter (top) and the control Gapdh coding region (bottom). Band intensities were determined using Image J and with the values for control samples from mice fed normal chow set to 1 (below the panel). Consistent results were observed from two re-ChIP assays. (B) HepG2 cells were treated with 50 mM of CDCA for 3 h and ChIP assays were performed. Band intensities were measured and the intensities relative to untreated samples were plotted with the SEM, n=3, indicated (right panel). (C) Mice were injected via tail veins with Ad-flag-SHP WT or the R57W mutant and 5 days later, were fed CA chow for 3 h. Livers were then collected for re-ChIP assays. Chromatin was immunoprecipitated with M2 antibody first, eluted, and then re-precipitated with the indicated antibody (left side), NS = normal serum. Semi-quantitative PCR was performed to detect occupancy of the proteins at the Cyp7a1, Cyp8b1, Srebp-1c promoters, and the Gapdh coding region as a control. Band intensities were determined using Image J and with values for samples from mice infected with Ad-SHP WT were set to 1 (below the panels).

Fig. 2.7

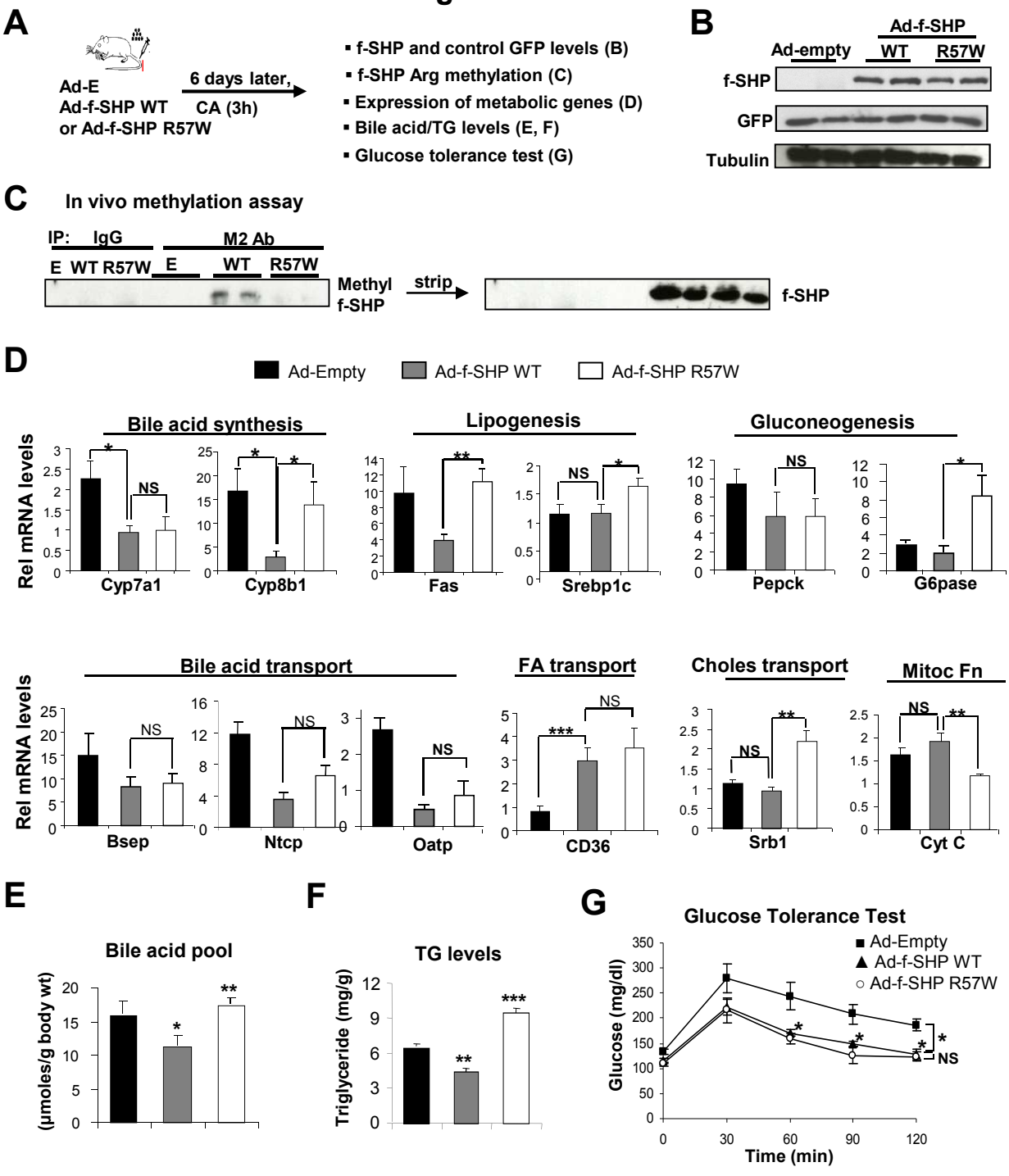

A
Ad-E
Ad-f-SHP WT
or Ad-f-SHP R57W
6 days later,
CA (3h)
▪ f-SHP and control GFP levels (B)
▪ f-SHP Arg methylation (C)
▪ Expression of metabolic genes (D)
▪ Bile acid/TG levels (E, F)
▪ Glucose tolerance test (G)
B
Ad-f-SHP
Ad-empty
WT
R57W
f-SHP
GFP
Tubulin
C
In vivo methylation assay
IP: IgG
M2 Ab
E WT R57W E WT R57W
Methyl f-SHP
strip
f-SHP
D
Ad-Empty
Ad-f-SHP WT
Ad-f-SHP R57W
Bile acid synthesis
Lipogenesis
Gluconeogenesis
Rel mRNA levels
Cyp7a1
Cyp8b1
Fas
Srebp1c
Pepck
G6pase
Bile acid transport
FA transport
Choles transport
Mitoc Fn
Bsep
Ntcp
Oatp
CD36
Srb1
Cyt C
NS
E
Bile acid pool
(μmoles/g body wt)
F
TG levels
Triglyceride (mg/g)
G
Glucose Tolerance Test
Ad-Empty
Ad-f-SHP WT
Ad-f-SHP R57W
Glucose (mg/dl)
Time (min)

Fig. 2.7. Hepatic overexpression of the methylation-defective R57W mutant reverses repression of known SHP metabolic targets in a gene-selective manner. (A) Experimental outline. (B) Protein levels in liver extracts were detected by western analysis. (C) Flag-SHP was immunoprecipitated and methylated SHP was detected by western analysis using SYM10 antibody in duplicate samples. (D) Expression of SHP target genes in different metabolic pathways was detected by q-RTPCR. The mean and SEM (n=5) are shown. (E, F) Total bile acid pool levels in liver, gall bladder, and intestines and liver triglyceride levels were measured (n=5). (G) Glucose tolerance tests in mice infected with control Ad-empty, Ad-SHP WT, or Ad-R57W (n=3-4). The mean and SEM is plotted. Statistical significance was measured using the Student's t test. *, **, ***, and NS indicate $p<0.05$, $p<0.01$, $p<0.001$, and statistically not significant, respectively.

Supplemental figures

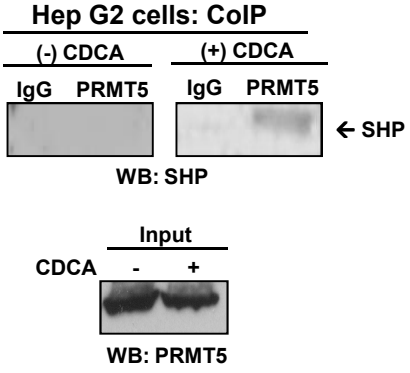
S2.1

Mutations identified in the human SHP gene among Japanese and European subjects

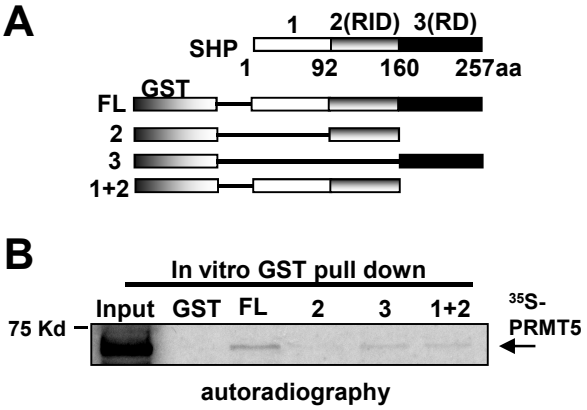
Mutation	Metabolic Disorder associated with SHP mutation			
	Early-onset obesity	High birth weight	Diabetes	Decreased insulin sensitivity
Mutations with reduced activity:				
R34X	✓	✓	✓	
H53fsdel10	✓	✓	✓	✓
R54C				
R57W	✓			
L98fsdel9insAC	✓		✓	✓
V105G				
G189E	✓			
A195S	✓		✓	✓
W206S				
R213C	✓	✓	✓	✓
Mutations with normal activity:				
R38C **R45P** G171A D178N **R216H**				

Adapted from Enya et al., *Human Mutation* 2008 Nov;29(11):E271-7

S2.2



S2.3



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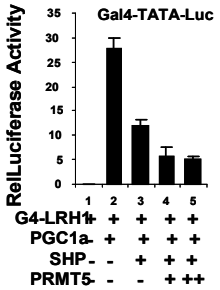
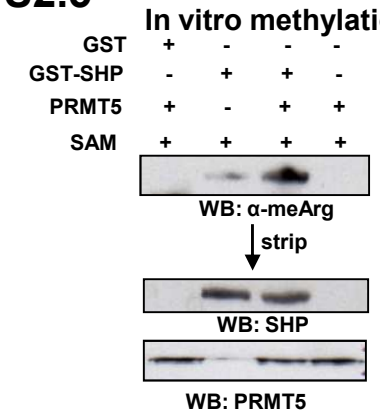


Supplemental figures (cont.)

S2.5

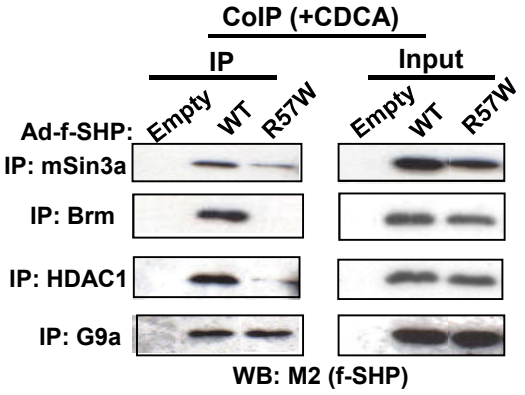


S2.6

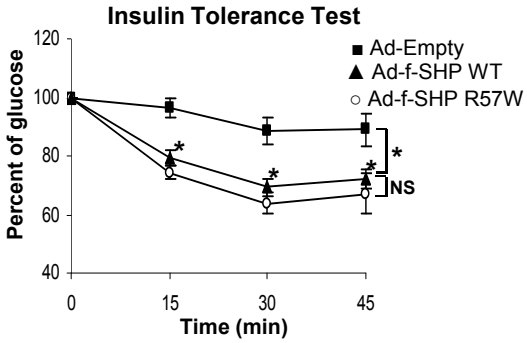
SHP sequences

Human	49 LCAPHRTC**R**EALDVLAKT 66
Rat	49 LCAPHRTC**R**EALDVLGKT 66
Mouse	49 LCAPHRTC**R**EALDVLAKT 66
Chimpanzee	49 LCAPHRTC**R**EALDVLAKT 66
Monkey	49 LCAPHRTC**R**EALDVLAKT 66
Horse	52 LCAPNRTC**R**EALDVLAKT 69
Cattle	52 LCTPHRTC**R**EALDVLAKT 69
Dog	52 LCAPHRTC**R**EALDVLART 69
Rabbit	52 LCSPHRTC**R**EALDVLAKT 69

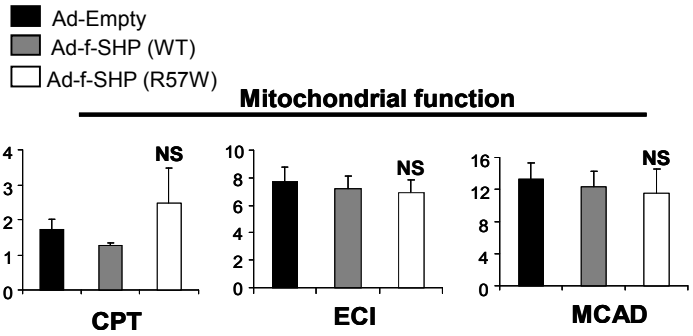
S2.7



S2.9



S2.8



Supplemental Figure legends

Fig. S2.1. Mutation identified in human SHP gene with metabolic disorders. A summary of human SHP mutations identified in Japanese and European subjects is shown. Metabolic diseases associated with them are also shown. Mutations occurring at Arg residues are indicated in bold character.

Fig. S2.2. Interaction between endogenous PRMT5 and SHP in HepG2 cells was increased after CDCA treatment. HepG2 cells were treated with CDCA or vehicle for 1 h. Cell lysates were prepared and immunoprecipitated with IgG or PRMT5 antibody and western blotted with anti-SHP.

Fig. S2.3. Direct interaction between SHP and PRMT5 in vitro. (A) Schematic diagrams of the receptor interacting domain (RID) and intrinsic repression domain (RID) in SHP are shown. (B) GST-SHP full length, deletion mutants, or control GST was incubated with 35S-labeled PRMT5 and in vitro GST pull down assays were performed. (C) Amounts of GST-SHP proteins or control GST used in the reaction were visualized by colloidal staining.

Fig. S2.4. Ectopic expression of PRMT5 augments SHP repression of LRH-1 transactivation of the Gal4 reporter activity. HepG2 cells were cotransfected with reporter plasmids, 200 ng of G4-TATA-luc reporter along with CMV b-galactosidase as an internal control, and expression plasmids, 25 ng of G4HNF-4 or 100 ng of G4-LRH1, 10 ng or 200 ng of PGC-1a respectively, 5 ng of pcDNA3-flag-SHP, 100 ng or 300 ng of PRMT5. Twenty-four h after transfection, cells were treated with CDCA overnight and reporter assays were performed. The values for firefly luciferase activities were normalized by dividing with the b-galactosidase activities, SEM (n=3).

Fig. S2.5. PRMT5 methylates GST-SHP in vitro. GST-SHP or control GST was incubated with purified PRMT5 and unlabelled SAM, and methylated SHP was detected using SYM10 methyl Arg antibody. SHP and PRMT5 levels were detected by western analysis.

Fig. S2.6. Arg 57, the arginine methylation site, is highly conversed in mammals. Alignment of the SHP region containing R57 from various species is shown.

Fig. S2.7. Interaction between endogenous SHP and G9a in HepG2 cells was increased after CDCA treatment. HepG2 cells were infected with Ad-flag-SHP and 24 hr later, cells treated with CDCA or vehicle for 1 h. Total cell lysates were prepared and immunoprecipitated with IgG or G9a antibody and western blotted with M2 antibody.

Supplemental Figure legends (cont.)

Fig. S2.8. Effects of hepatic overexpression of SHP wild type (WT) or R57W on expression of metabolic genes, CPT, ECI, or MCAD. Mice were tail vein injected with Ad-empty, Ad-flag-SHP WT, or Ad-flag-R57W and 6 days later, mice were fed 0.5% cholic acid supplemented chow for 3 h and livers were collected for performing q-RTPCR to measure mRNA levels of potential SHP target genes in liver. The mean and SEM (n=5) are shown. NS indicates statistically not significant.

Fig. S2.9. Mutation of Arg-57 does not change insulin tolerance in mice. Insulin tolerance tests in mice infected with control Ad-empty, Ad-SHP WT, or Ad-R57W (n=3-4). The mean and SEM is plotted. Statistical significance was measured using the Student's t test and * and NS indicate $p<0.05$ and statistically not significant, respectively.

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Chapter Three

Delineating the upstream signaling pathway controlling SHP methylation in health and disease

Abstract

Cellular signaling cascades regulate the activity of transcription factors in order to convert extracellular information into gene regulation. Small Heterodimer Partner (SHP) is a transcriptional repressor of several nuclear receptors involved in diverse cellular processes. We previously reported that in response to bile acids, SHP is post-translationally methylated by PRMT5 at Arg-57, which is critical for interaction with corepressors and repression of target genes. Consistently, a naturally-occurring R57W mutant associated with obesity in human subjects showed impaired repression of target genes. We now show that SHP is post-translationally phosphorylated at Thr-55 in response to bile acids. Assays using pharmacological inhibitors showed that a phosphoinositide-3-kinase (PI3K) and protein kinase C zeta (PKC zeta) signaling cascade is likely involved. Phosphorylation of SHP at Thr-55 is upstream of Arg-57 methylation, and thereby couples the PI3K-PKCζ signaling to arginine methylation. The phosphorylation-deficient mutant, T55A, as well as methylation-deficient mutant, R57K, showed reduced phosphorylation and methylation, suggesting that phosphorylation and methylation of SHP are interdependent. In addition, a phosphorylation-mimic mutant, T55D, showed increased methylation and activity compared to wild type. Targeting post-translational modifications of SHP may be an effective strategy to treat SHP-related human diseases, such as metabolic syndrome, cancer, infertility and inflammatory processes.

Introduction

SHP is an atypical orphan nuclear receptor since it lacks the conserved DNA-binding domain but contains a putative ligand-binding domain. Since its discovery, SHP has been identified as a key transcriptional repressor of genes involved in diverse biological processes, including metabolic pathways, cell proliferation, apoptosis and sexual maturation (1-9). SHP plays a major role in the negative feedback repression of bile acid biosynthesis in the liver through inhibition of the transcription of the key bile acid biosynthetic gene, CYP7A1. The role of SHP in the regulation of bile acid synthesis, fatty acid synthesis, and glucose production in response to bile acid signaling are well-established.

As signaling molecules, bile acids are known to activate nuclear receptors such as FXR (10-12), PXR, and VDR, G protein coupled receptors (GPCRs) such as TGR5 (12-15), as well as cell signaling pathways including the three mitogen-activated protein kinase (MAPK) signaling pathways (ERK, JNK and p38 MAPK), AKT/PKB and PKC (16-18). In response to elevated bile acids, induction of SHP gene expression by bile acid activated FXR is well established. The bile acid activated ERK pathway in the liver regulates genes controlling cell proliferation, survival and apoptosis, such as MYC, MYCN, STAT or ETS. Bile acids have been reported to induce energy expenditure by promoting thyroid hormone activation through the activation of the TGR5-signaling pathway (15). Recent studies have also linked bile acid signaling to cell proliferation and apoptosis through TGR5-mediated activation of the receptor tyrosine kinase EGFR (19) and JNK (20) signaling pathways. Bile acids also directly activate EGFR, which in turn activates the phosphoinositide-3-kinase (PI3K)-AKT pathway involved in regulation of gluconeogenesis (21, 22). Bile acids have also been shown to modulate cellular activity by increasing PKC phosphorylation and translocation (23, 24). However the role of bile acid

activated signaling pathways in direct modulation of the activity of nuclear receptors and transcription factors have not been reported.

We recently reported that bile acid signaling activates post-translational modifications (PTMs) of SHP, which play an important role in regulation of its activity and stability. Bile acid signaling activates ERK, which phosphorylates SHP at Ser-26, which increases SHP stability by inhibiting ubiquitination at Lys-122 and Lys-123 (25). Bile acid signaling also activates methylation of SHP by PRMT5 at Arg-57, which is critical for SHP repression activity. However, the upstream signaling pathway that controls methylation of SHP at Arg-57 in response to bile acids is not known.

By using pharmacological inhibitors and siRNA silencing, we demonstrate that a signaling pathway involving phosphoinositide 3 kinase (PI3K) and its downstream target PKC ζ, is involved in regulating arginine methylation of SHP. In response to bile acid signaling, PKC ζ phosphorylates SHP, which regulates the methylation of SHP by PRMT5. We show that both phosphorylation of SHP by PKC ζ and methylation by PRMT5 are critical for SHP repression activity.

Materials and Methods

Materials and Reagents

M2 antibody was purchased from Sigma, dimethyl symmetric Arg (SYM10) antibody from Upstate Biotech, phospho-Threonine antibody from Cell Signaling technology and mouse IgG antibody from Santa Cruz Biotech. Pharmacological kinase inhibitors were purchased from Calbiochem.

Construction of plasmids and adenoviral vectors

The expression plasmids, pcDNA3 flag-R57W, R57K, T55A and T55D mutants were generated using QuikChange site-directed mutagenesis kit (Stratagene) and positive clones were identified by DNA sequencing. For constructing Ad-flag-human SHP wild type and R57W mutant adenoviral vectors, the 0.9 kb Xba1 fragment from pCDNA3-flagSHP plasmid was inserted into Xba1-digested Ad-Track-CMV vector.

Cell cultures and transfection reporter assay

HepG2 cells (ATCC HB8065) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium. Cos-1 cells were maintained in DMEM medium. Cells were transfected with plasmids or infected with adenoviral vectors, incubated with serum-free media overnight, and treated with 50 μM CDCA for times indicated in the figure legends.

Mass spectrometry analyses

Flag-human SHP was expressed in HepG2 cells (three 15-cm plates per group) by adenoviral infection and 48 h later, cells were treated with 5 μM MG132 for 4 h to inhibit degradation and then further treated with CDCA for 1 h. Flag-SHP was isolated in RIPA (SDS) lysis buffer using M2 agarose. Proteins were separated by SDS-PAGE, visualized with colloidal staining and flag-SHP bands were excised, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Results

SHP is phosphorylated at Thr 55 in response to bile acid signaling

In order to determine the functional roles of post-translational modifications of SHP in response to bile acid signaling, modified sites were identified using tandem mass spectrometry (MS/MS) (Fig. 3.1A, B). We previously reported the identification of a SHP methylation site, Arg-57, in response to bile acid signaling. Now we report the identification of Thr-55 as a site of phosphorylation in SHP after CDCA treatment in HepG2 cells by tandem mass spectrometry (Fig, 3.1A, B, Fig. S3.1). Thr-55 is highly conserved in mammals (Fig. 3.1C). Phosphorylation of wildtype SHP was dramatically increased by CDCA treatment of HepG2 cells, whereas mutation of Thr-55 to alanine abolished the phosphorylation of SHP (Fig. 3.1D), confirming that Thr-55 is the major site of phosphorylation. In addition to bile acids, FGF19 and insulin signaling pathways also dramatically increased the Thr-phosphorylation of SHP, but a selective agonist for FXR, GW4064, had no effect on SHP phosphorylation in either HepG2 cells (Fig. 3.1E) or primary human hepatocytes (Fig. S3.2). These proteomic and biochemical studies demonstrate that SHP is phosphorylated at Thr-55 and suggest that bile acid signaling pathways substantially increase SHP phosphorylation.

An atypical PKC, PKCζ, is involved in SHP phosphorylation

In order to identify the kinase involved in phosphorylation of SHP at Thr-55, we used pharmacological inhibitors of specific kinases. Inhibition of the MAP kinases, ERK1/2, JNK or p38 MAPK, or AKT/PKB had no effect on Thr-phosphorylation of SHP (data not shown). Inhibition of the PKC isoforms showed that none of the classical or novel PKCs was involved. A

myristoylated pseudosubstrate inhibitor of PKC ζ and a pseudosubstrate inhibitor of PKC ζ/ι substantially decreased the phosphorylation of SHP, suggesting that the atypical PKC, PKC ζ, is involved in SHP Thr-phosphorylation (Fig. 3.1F). Abolished phosphorylation of SHP by inhibition of phosphoinositide-3-kinase (PI3K) by wortmannin suggested that PI3K is also present in the signaling pathway of SHP phosphorylation (Fig. 3.1F). Decreased methylation levels of SHP by inhibitors of PKC ζ and PI3K (Fig. 3.1F, bottom panel) suggested that the PI3K-PKC ζ signaling pathway also regulates methylation of SHP.

Thr-55 phosphorylation augments SHP repression function

To test the functional relevance of Thr-55 phosphorylation, the activity of the phosphorylation-deficient SHP mutant, T55A, was examined by cell-based reporter assays. Repression of the transactivation activity of HNF-4 (Fig. 3.2A) and LRH1 (Fig. 3.2B) by SHP was substantially abrogated by the T55A mutant when compared to wild type (WT) SHP. The repression effects of SHP were also markedly reduced by the methylation-deficient R57K mutant (Fig. 3.2A, B). A phosphorylation-mimic mutant of SHP, T55D, showed enhanced repression of HNF4 activity, supporting the conclusion that phosphorylation of SHP at Thr-55 is critical for SHP repression activity (Fig. 3.2C).

Phosphorylation of SHP enhances SHP arginine methylation

The close proximity of the phosphorylation and methylation sites (T55 and R57), and the abolished activation of phosphorylation and methylation of SHP by inhibition of PI3K and PKC ζ (Fig. 3.1F), suggested the possibility of cross-talk between SHP phosphorylation and methylation. To test this, the phosphorylation and methylation levels of T55A and R57K mutants were examined. Both mutants showed significantly reduced phosphorylation and methylation

levels compared to WT (Fig. 3.3A). In addition, the phosphorylation-mimic T55D mutant showed increased methylation levels compared to WT (Fig. 3.3B). These results strongly suggested that phosphorylation and methylation of SHP are interdependent. Time-dependent CDCA or FGF19 treatment showed that SHP is phosphorylated within 15 min of ligand treatment (Fig. 3.3C, D), suggesting that the phosphorylation of SHP is a rapid response to bile acid signaling.

Discussion

Our studies demonstrate that phosphorylation and methylation of SHP cooperatively modulate SHP activity, and that decreased levels of methylated and phosphorylated SHP are present in a mouse model of metabolic disease. First, tandem mass spectrometry and proteomic studies showed that phosphorylation of SHP at Thr-55 is substantially increased after bile acid treatment. Secondly, assays with pharmacological kinase inhibitors revealed that a signaling pathway involving PI3K and PKC ζ is involved in SHP phosphorylation, and phosphorylation also regulates arginine methylation of SHP. Thirdly, studies with the phosphorylation-defective and methylation-defective mutants showed that there is interplay between phosphorylation and methylation of SHP, and that they are interdependent.

The function of bile acids as signaling molecules in activation of cellular signaling pathways has recently been demonstrated (16-18). Bile acids have been shown to activate nuclear receptors such as FXR (10-12), PXR and VDR, G protein coupled receptors (GPCRs) such as TGR5 (12-15), as well as cell signaling pathways including that of mitogen-activated

protein kinases (MAPKs) (ERK, JNK and p38 MAPK), AKT/PKB and PKC (16-18). However the role of bile acids in directly modulating the activity and/or stability of nuclear receptors or transcriptional factors is not very clear. We recently reported that bile acid signaling activates ERK, which phosphorylates SHP at Ser-26, which increases SHP stability in hepatocytes (25). We also showed that bile acid signaling activates methylation of SHP, which increases SHP activity. This is the first study to demonstrate that SHP repression activity is increased by posttranslational modifications mediated by a bile acid activated kinase signaling pathway. The role of PKC ζ in phosphorylation and enhanced transactivation ability of FXR was reported by Schneider et al. (26). However, whether bile acids increase the PKC ζ effect on FXR activity is not known.

PTMs, such as methylation, ubiquitination, and phosphorylation, subtly or sometimes dramatically, regulate the activity and/or stability of cellular regulatory proteins. Because these modifications are reversible processes, activity and/or stability of regulatory proteins can be modulated dynamically in response to cellular signals. Given that SHP plays important roles in such diverse mammalian physiology, PTMs may provide a mechanism of selective regulation of genes in biological processes. Further, targeting post-translational modifications of SHP may be an effective therapeutic strategy by controlling selected groups of genes to treat SHP-related human diseases, such as metabolic syndrome, cancer, and infertility. Also, it will be important to determine whether decreased PTMs of SHP, which results in decreased SHP activity are associated with metabolic disease, if components of the pathways involved in the regulation of SHP activity may be attractive targets for the development of therapeutic agents that modulate SHP activity to treat metabolic disorders.

Figures and legends

Fig. 3.1

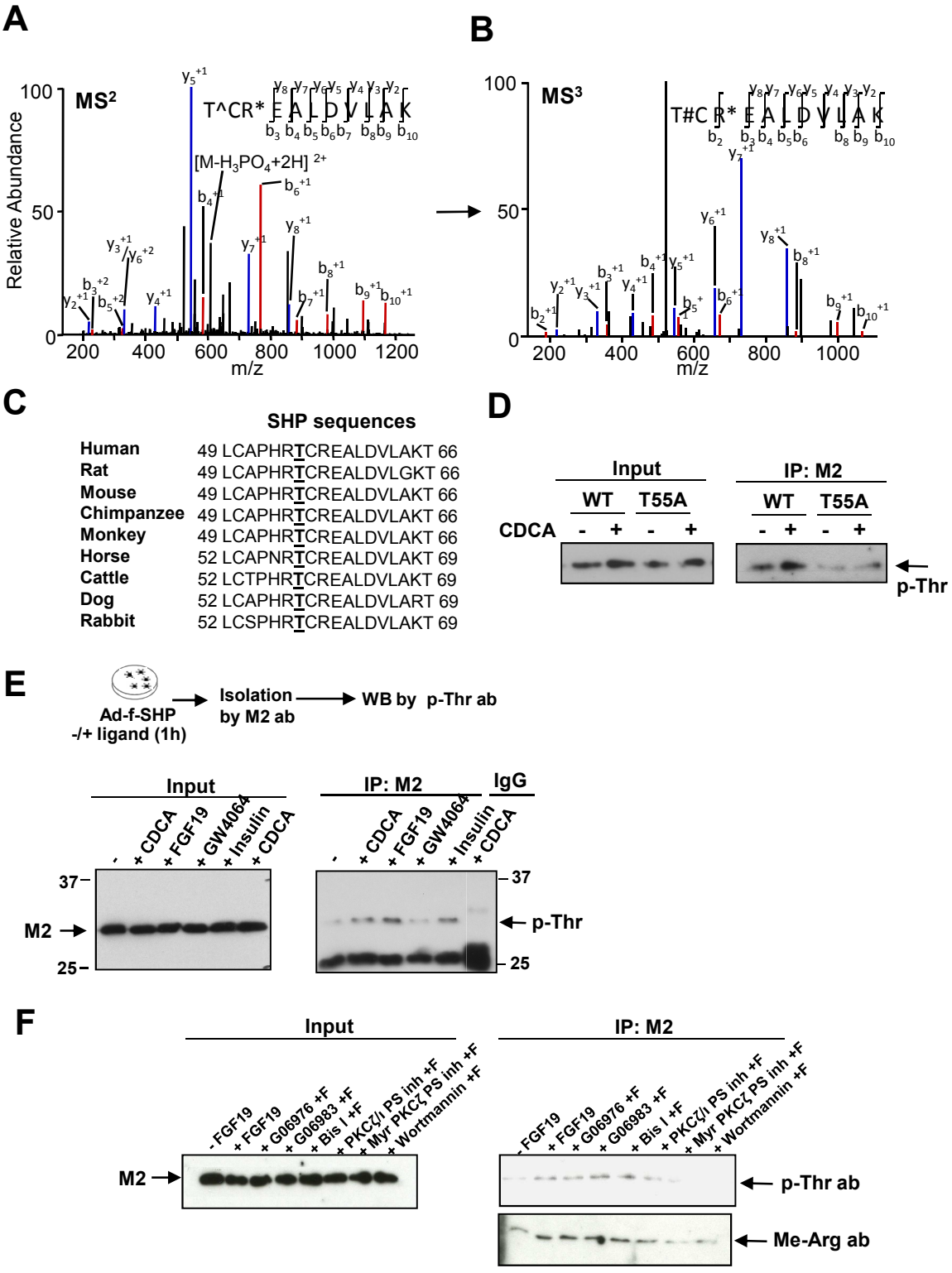

Fig. 3.1. SHP is phosphorylated at Thr-55 in response to bile acid signaling, and an atypical PKC, PKC ζ, is likely involved in SHP phosphorylation
(A, B) The MS/MS spectrum of the SHP peptide containing phosphorylated Thr-55 and methylated Arg-57. The MS^2 scan in A was followed by the MS^3 scan in B, which added confirmation to phosphorylation at Thr-55. (C) Alignment of the SHP region containing Thr-55 from various species is shown. (D) HepG2 cells were transfected with pCDNA3-flag-SHP WT or T55A, treated with vehicle or CDCA for 1 h, and flag-SHP was immunoprecipitated by M2 antibody, and phosphorylated SHP was detected by western analysis using p-Thr antibody. (E) HepG2 cells were infected with Ad-flag-SHP WT, treated with the indicated ligands for 1 h, and flag-SHP was immunoprecipitated by M2 antibody, and phosphorylated SHP was detected by p-Thr antibody. (F) HepG2 cells were infected with Ad-flag-SHP WT, treated with the indicated kinase inhibitors for 1 h, followed by treatment with FGF19 for 1 h. Flag-SHP was immunoprecipitated by M2 antibody, and phosphorylated SHP was detected by p-Thr antibody, and methylated SHP by SYM10 antibody.

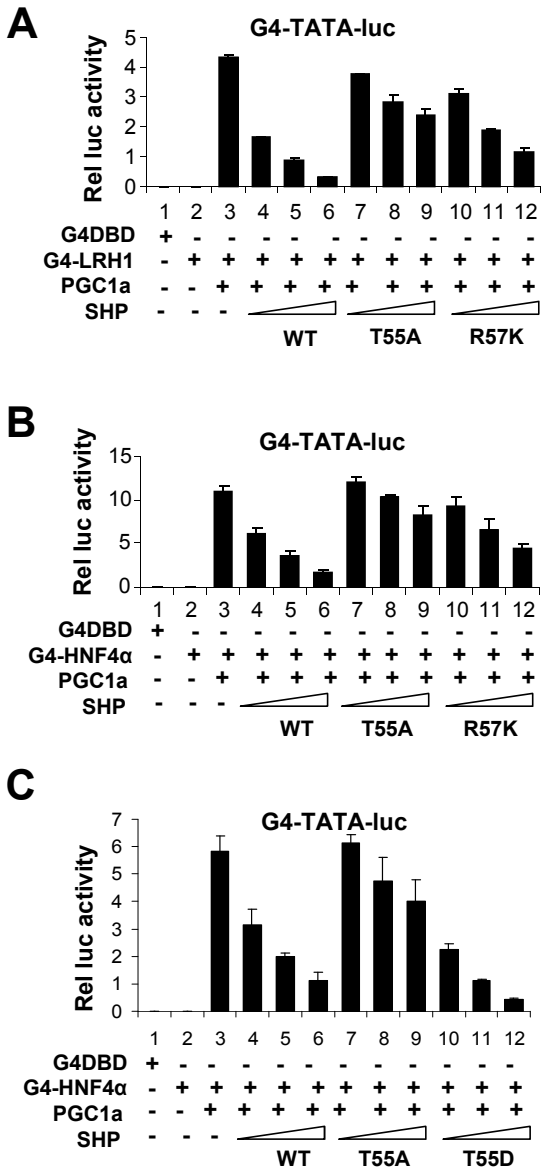

Fig. 3.2. Thr-55 phosphorylation augments SHP repression function
HepG2 cells transfected with plasmids as indicated (for plasmid amounts, see Materials and Methods) were treated with CDCA overnight and reporter assays were performed. The triangles represent increasing amounts of the flag-SHP vectors. The values for firefly luciferase activities were normalized by dividing by the β-galactosidase activities. The mean and SEM is plotted (n=3).

Fig. 3.3

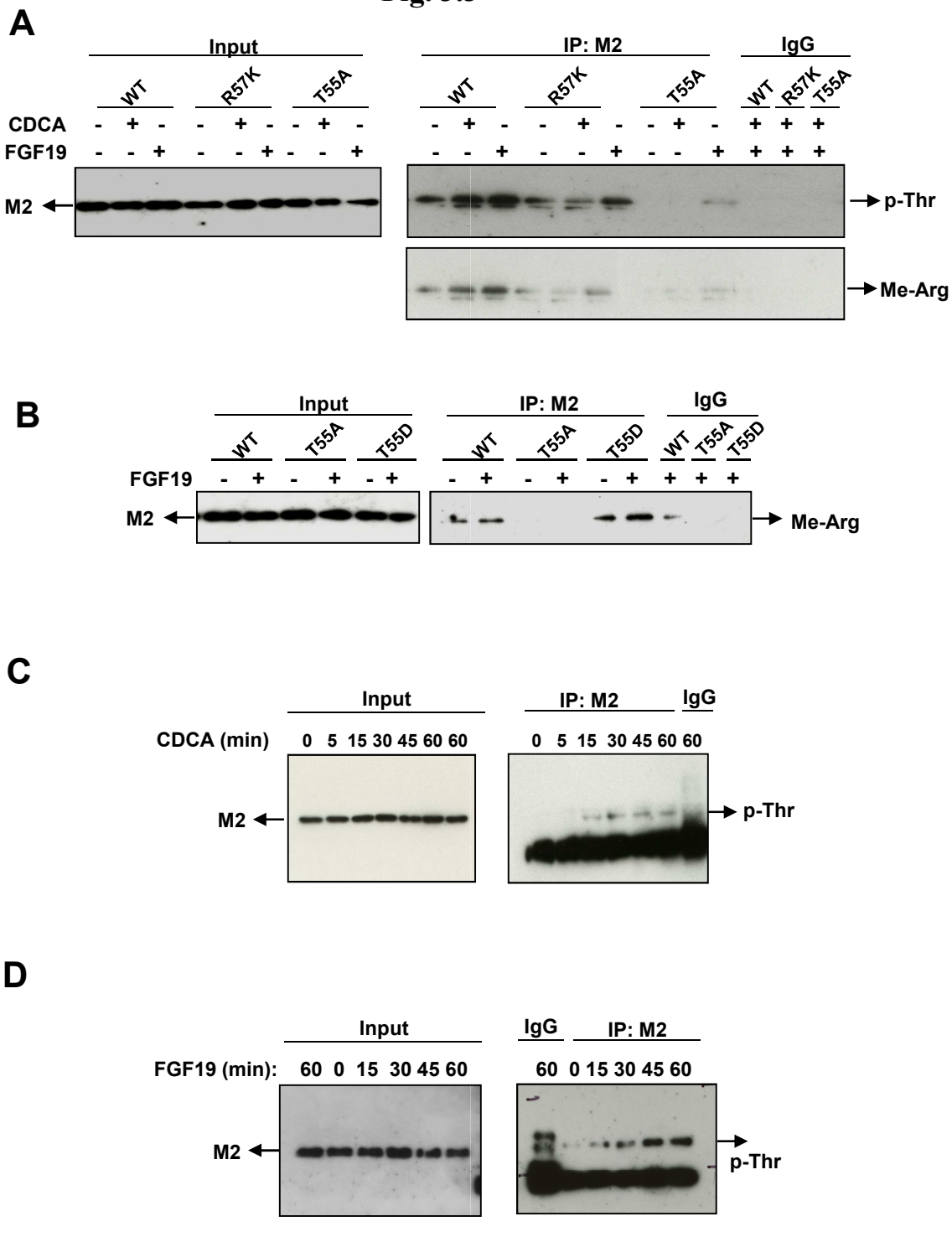
A
Input
IP: M2
IgG
WT
R57K
T55A
CDCA
FGF19
M2
p-Thr
Me-Arg
B
Input
IP: M2
IgG
WT
T55A
T55D
FGF19
M2
Me-Arg
C
Input
IP: M2
IgG
CDCA (min)
0 5 15 30 45 60 60
M2
p-Thr
D
Input
IgG
IP: M2
FGF19 (min):
60 0 15 30 45 60
60 0 15 30 45 60
M2
p-Thr

Fig. 3.3. Crosstalk between phosphorylation and arginine methylation of SHP (A) HepG2 cells transfected with pCDNA3-flag-SHP WT, pCDNA3-flag-SHP R57K or pCDNA3-flag-SHP T55A were treated with the indicated ligands for 1 h, flag-SHP was immunoprecipitated by M2 antibody, and phosphorylated SHP was detected by p-Thr antibody, and methylated SHP by SYM10 antibody. (B) HepG2 cells transfected with pCDNA3-flag-SHP WT, pCDNA3-flag-SHP T55A or pCDNA3-flag-SHP T55D were treated with FGF19 for 1 h, flag-SHP was immunoprecipitated by M2 antibody, and methylated SHP by SYM10 antibody. (C, D) HepG2 cells infected with Ad-flag-SHP WT were treated with CDCA (C) or FGF19 (D) for the indicated time points, flag-SHP was immunoprecipitated by M2 antibody, and phosphorylated SHP was detected by p-Thr antibody.

Supplemental figures

S3.1

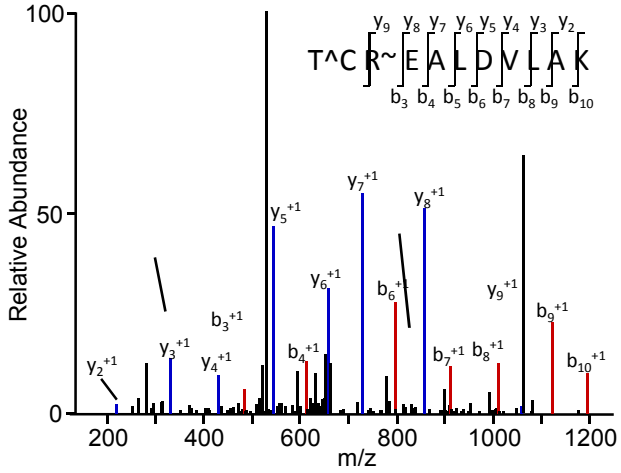

Fig. S3.1. SHP is phosphorylated at Thr 55 and methylated at Arg 57 in response to bile acid signaling The MS/MS spectrum of the SHP peptide containing phosphorylated Thr-55 and trimethylated Arg-57.

S3.2

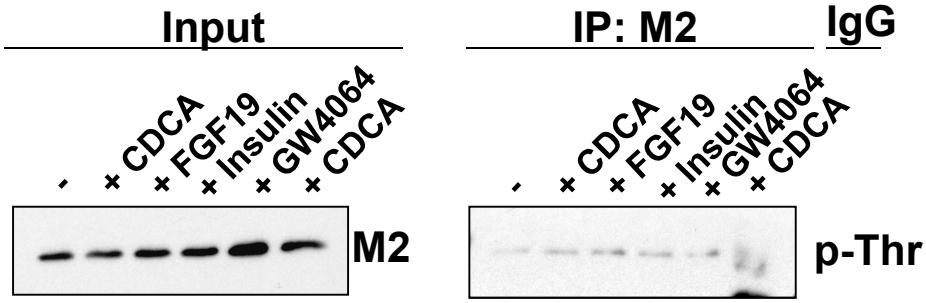

Fig. S3.2. Primary human hepatocytes were infected with Ad-flag-SHP WT, treated with the indicated ligands for 1 h, and flag-SHP was immunoprecipitated by M2 antibody, and phosphorylated SHP was detected by p-Thr antibody.

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Chapter Four

Discussion and conclusions

The main purpose of this study is to understand the molecular mechanisms by which the activity of SHP is regulated by post-translational modifications, and whether the activity of SHP is abnormally regulated in metabolic disease state. We demonstrate in these studies that methylation and phosphorylation of SHP modulate its activity and that decreased levels of phosphorylated and methylated SHP are present in a mouse model of metabolic disease. In response to bile acid signaling, the interaction of SHP with PRMT5 is increased and SHP is methylated by PRMT5 at Arg-57. Mutation of Arg-57 resulted in reduced repression activity and decreased interaction with cofactors of SHP, Brm, mSin3A, and HDAC1, but not with G9a, and their subsequent recruitment to SHP target genes. Overexpression of the methylation-defective R57W mutant or downregulation of PRMT5 in mouse liver led to reduced repression of SHP metabolic target genes in a gene-selective manner. Consistent with gene expression patterns, the overexpression of Arg-57 mutant of SHP led to elevated bile acid pool size and liver triglyceride levels, but interestingly, the effects on glucose and insulin tolerance were not altered from WT SHP.

Naturally-occurring heterozygous mutations, including R57W, in the SHP gene have been reported in human subjects with obesity, type II diabetes, and fatty liver (1-5), which indicates the critical role of SHP in metabolic regulation. The effects of the R57W mutation on gene expression, and triglyceride and bile acid levels in mice are consistent with its association with human metabolic disease. Hepatic expression of the R57W mutant markedly increased the

transcription of lipogenic and bile acid synthetic genes in comparison to that of wild type SHP. These changes in gene expression patterns were consistent with elevated hepatic triglyceride levels and total bile acid pool. In addition, conformational changes in R57W contributed to more severe effects on the reduced repression activity of SHP since the more conservative R57K mutation resulted in milder effects on SHP activity. Taken together, these results provide a possible explanation of why the R57W mutation is associated with metabolic syndrome in humans.

In this study, we identified the upstream signaling pathway that regulates SHP methylation. In response to bile acid signaling, SHP is phosphorylated at Thr 55. A signaling pathway involving PI3K and PKC ζ is involved in this SHP phosphorylation that also regulates the downstream methylation of SHP. The role of PKC ζ in phosphorylation and enhanced transactivation activity of FXR has been reported (6). However, whether bile acids increase the PKC effect on FXR activity is not known. FGF15/19 signaling, which is activated in response to elevated bile acids, and insulin signaling also increased the Thr-phosphorylated levels of SHP. Mutation of Thr-55 resulted in reduced SHP repression activity, indicating that Thr-55 phosphorylation of SHP is critical for its activity. Both T55A and R57K mutants showed reduced phosphorylated and methylated levels, suggesting that phosphorylation at T55 and methylation at R57 are interdependent. SHP was rapidly phosphorylated in response to bile acid and FGF19 signaling pathways.

Based on our studies, we propose a model for the regulation of SHP activity in response to bile acid and FGF19 signaling pathways (Fig. 4.1). Bile acids and FGF19 activate kinase

signaling pathways, which activate PI3K and subsequently, its downstream target PKC ζ. PKC ζ in turn activates the phosphorylation of SHP, which increases its interaction with PRMT5 and methylation at Arg-57. PTMs alter SHP structure to favour binding of corepressors, which leads to SHP-mediated repression of target genes in a gene-specific manner.

SHP is emerging as a critical regulator for multiple metabolic pathways, including cholesterol/bile acid, fatty acid/triglyceride, and glucose metabolism (7-9). Dysregulation of these metabolic pathways underlies major metabolic diseases in humans, such as liver steatosis, obesity, diabetes, and cardiovascular disease. Our studies show that regulation of SHP activity is important both in physiological regulation of metabolic homeostasis and in pathophysiological conditions. Given that SHP plays an important role in diverse biological programs, modulating activity of SHP may provide new pharmacological options for the treatment of human metabolic diseases. SHP was discovered as an orphan receptor (10) and an endogenous ligand of SHP has not been identified. In this regard, modulation of SHP activity by PTM in response to physiological stimuli would be an alternative effective way to control its activity and/or stability. SHP is a well known component of cellular sensors for bile acid signaling (7, 11). We recently reported that SHP undergoes rapid degradation with a half-life of about 30 min and bile acid signaling activates ERK, which phosphorylates SHP at Ser-26 and increases SHP stability (12). Thus, in addition to SHP gene induction by the bile acid-activated nuclear receptor FXR, modulation of SHP stability and activity by PTMs is likely to be important in the mediation of bile acid signaling by SHP. It will also be important to determine whether decreased PTMs of SHP, which results in decreased SHP activity are associated with metabolic disease. If so,

components of the pathways involved in the regulation of SHP activity may be attractive targets for the development of therapeutic agents that modulate SHP activity to treat metabolic disorders.

Figures and legends

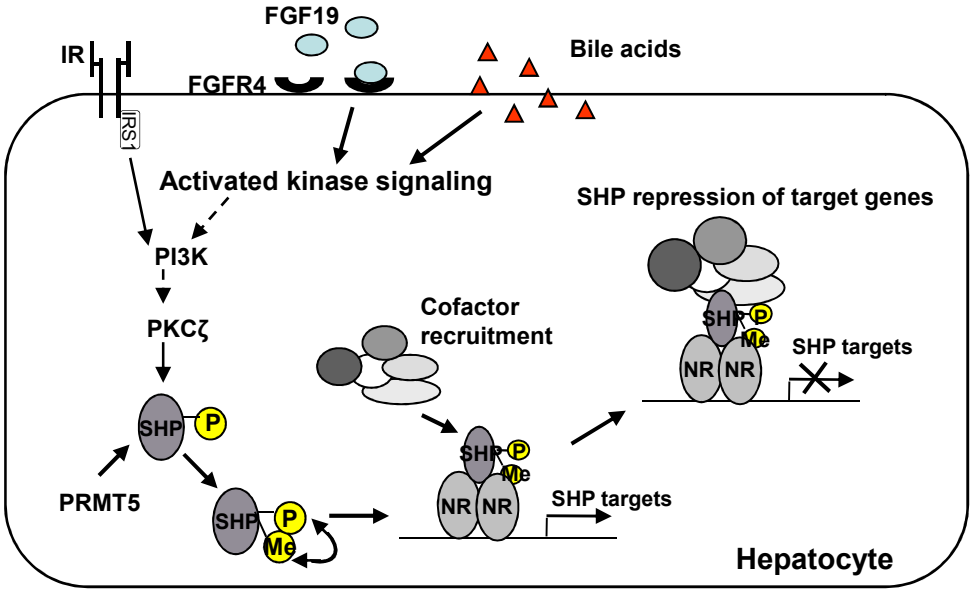

Fig. 4.1 Proposed model for the regulation of SHP activity by posttranslational modifications in response to bile acid signaling pathways Bile acids, FGF19 and insulin activate a common downstream target, PI3K, which in turn activates its downstream target PKC ζ. PKC ζ in turn activates the phosphorylation of SHP at Thr-55, which increases its interaction with PRMT5 and methylation at Arg-57. PTMs provide distinct protein interaction interfaces that facilitate recruitment of cofactors, which leads to SHP-mediated repression of target genes.

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