Inhibition of protein arginine methyltransferase 5 enhances hepatic mitochondrial biogenesis

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Protein arginine methyltransferase 5 (PRMT5) regulates gene expression either transcriptionally by symmetric dimethylation of arginine residues on histones H4R3, H3R8, and H2AR3 or at the posttranslational level by methylation of nonhistone target proteins. Although emerging evidence suggests that PRMT5 functions as an oncogene, its role in metabolic diseases is not well-defined. We investigated the role of PRMT5 in promoting high-fat–induced hepatic steatosis. A high-fat diet up-regulated PRMT5 levels in the liver but not in other metabolically relevant tissues such as skeletal muscle or white and brown adipose tissue. This was associated with repression of master transcription regulators involved in mitochondrial biogenesis. In contrast, lentiviral short hairpin RNA–mediated reduction of PRMT5 significantly decreased phosphatidylinositol 3-kinase/AKT signaling in mouse AML12 liver cells. PRMT5 knockdown or knockout decreased basal AKT phosphorylation but boosted the expression of peroxisome proliferator–activated receptor α (PPARα) and PGC-1α with a concomitant increase in mitochondrial biogenesis. Moreover, by overexpressing an exogenous WT or enzyme-dead mutant PRMT5 or by inhibiting PRMT5 enzymatic activity with a small-molecule inhibitor, we demonstrated that the enzymatic activity of PRMT5 is required for regulation of PPARα and PGC-1α expression and mitochondrial biogenesis. Our results suggest that targeting PRMT5 may have therapeutic potential for the treatment of fatty liver.

The phenotypic spectrum of nonalcoholic fatty liver disease (NAFLD)[3] is broad and may range from simple steatosis to ste-atohepatitis, advanced fibrosis, and cirrhosis. NAFLD is commonly associated with type 2 diabetes, visceral obesity, and hyperlipidemia (1). There is evidence that increased delivery of nonesterified fatty acid to the liver (mainly from white adipose tissue (WAT)) is fundamental to the development of NAFLD (2, 3). The role of diet in the pathogenesis of NAFLD has been investigated both in humans and in animal models (4–6). Subjects on a high-fat diet develop fatty liver (5, 6); those on a low-fat/high-carbohydrate diet develop fatty liver via increased *de novo* fatty acid synthesis (4). In addition, high dietary fat intake is associated with obesity and insulin resistance. As a consequence of insulin resistance, suppression of lipolysis by insulin is impaired, leading to increased nonesterified fatty acid delivery to the liver (7, 8). Thus, high dietary fatty acids have profound effects on insulin resistance, NAFLD, and cardiovascular disease (9).

Phosphatidylinositol 3-kinase (PI3K), a lipid kinase, generates the second messenger phosphatidylinositol 1,4,5-trisphosphate (10). The accumulation of phosphatidylinositol 1,4,5-trisphosphate recruits protein kinase B (AKT) to the cell membrane, where it is phosphorylated and activated by phosphoinositide-dependent kinase 1 and 2 (11, 12). Downstream of this PI3K/AKT pathway is mTOR, a major effector that is specifically implicated in the regulation of cell growth via nutrient availability and cellular bioenergetics (13). Activation of the PI3K–AKT–mTOR pathway is accompanied by aberrant lipid metabolism in cancers (14), where it increases the protein levels of sterol regulatory element–binding proteins (SREBPs) and its target, fatty acid synthase (FASN) (15, 16). Inhibition of the mTOR pathway is associated with impaired induction of transcription factors, *i.e.* peroxisome proliferator activated receptor α (PPARα) and its target genes, such as carnitine palmitoyltransferase 1 (CPT1). Under conditions favoring re-esterification over β-oxidation in the liver, newly synthesized triglycerides are carried on very-low-density lipoproteins (VLDLs) to be redistributed to WAT for storage or to skeletal muscle for uti-

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[3] The abbreviations used are: NAFLD, nonalcoholic fatty liver disease; WAT, white adipose tissue; PI3K, phosphatidylinositol 3-kinase; SREBP, sterol regulatory element–binding protein; FASN, fatty acid synthase; PPAR, per-oxisome proliferator–activated receptor; VLDL, very-low-density lipoprotein; shRNA, short hairpin RNA; HFD, high-fat diet; RD, regular diet; MEF, mouse embryonic fibroblast; 4-OHT, 4-hydroxytamoxifen; DMEM, Dulbecco's modified Eagle's medium; ChIP-seq, ChIP sequencing; cDNA, complementary DNA; DAPI, 4′,6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT-qPCR, quantitative real-time PCR.

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lization (under exercise conditions). Inhibition of PI3K/AKT can abolish insulin-induced suppression of VLDL assembly and secretion by regulating the degradation of apolipoprotein B (ApoB) and the gene expression of hepatic microsomal triglyceride transfer protein (17). Thus, the PI3K–AKT–mTOR signaling pathway plays an integral role in regulating several pathways contributing to lipid homeostasis in the liver, including *de novo* lipid biosynthesis, fatty acid β-oxidation (18, 19), and VLDL assembly and secretion.

The protein arginine methyltransferase family consists of three subfamilies that differ in their ability to carry out monomethylation or asymmetric dimethylation (type I), monomethylation or symmetric dimethylation (type II), or exclusively monomethylation (type III) (20, 21). PRMT5 functions as the major type II arginine methyltransferase that symmetrically dimethylates arginine residues on histones as well as nonhistone proteins, including RNA splicing factors and cell cycle regulators (22, 23). PRMT5-mediated methylation of histones H2AR3 (24), H4R3 (25), and H3R8 (26) regulates chromatin structure and influences transcription. We have shown that PRMT5 is required for activation of adipogenic gene expression by promoting adipogenic master transcriptional factor PPARγ2 expression during pre-adipocyte differentiation (27, 28). PRMT5 has been shown to methylate SREBP1a on Arg-321 and increases SREBP1a transcriptional activity, thereby promoting *de novo* lipogenesis and tumor growth (29). Overexpressing exogenous GFP-PRMT5 resulted in elevated levels of phospho-AKT in 293T cells (30). However, little is known about the role of endogenous PRMT5 in regulating lipid homeostasis in the context of NAFLD.

In this study, we investigated the function of PRMT5 in a diet-induced obesity mouse model. We found that a high-fat diet increased PRMT5 expression in the liver but not in other metabolically relevant tissues. Hepatic PRMT5 expression, however, was not altered in the liver of the obese Agouti mouse, suggesting that high-fat diet consumption, but not obesity *per se*, induced PRMT5 expression. Global signaling pathway analysis showed that knockdown of PRMT5 attenuated PI3K/AKT signaling. In the nontransformed mouse liver cell line model α mouse liver 12 (AML12), reducing PRMT5 levels by shRNA or inhibiting its enzymatic activity with a small-molecule inhibitor led to an increase in PPARα and PGC-1α levels and mitochondrial biogenesis. Our results suggest that inhibiting PRMT5 favors β-oxidation, which would contribute to the reversal of hepatic steatosis.

Results

PRMT5 is ubiquitously expressed in mice

Although PRMT5 is ubiquitously expressed at varying levels in all mouse tissues examined, its expression is most abundant in the spleen and kidneys, and it is expressed in tissues important for metabolic control, such as liver, muscle, brown adipose tissue, and inguinal WAT (Fig. 1*A*). Similarly, PRMT5 was detected in the cytoplasm as well as in the nucleus of the nontransformed AML12 murine hepatocyte cell line (Fig. 1*B*).
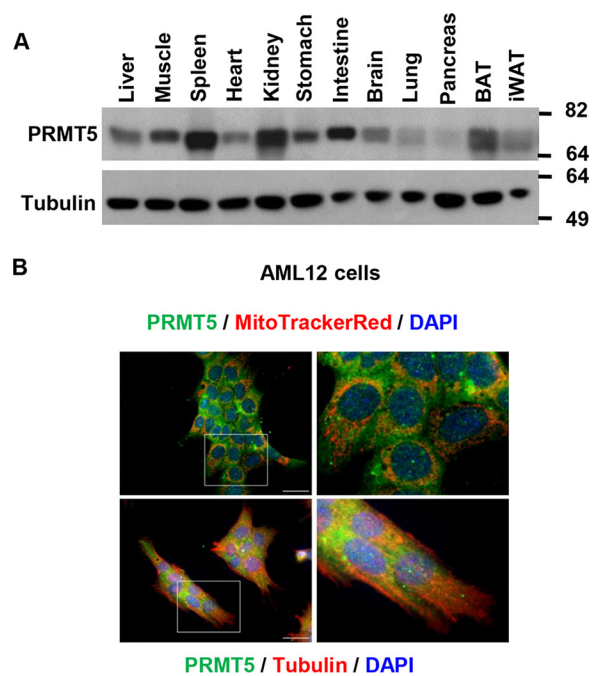

Figure 1. PRMT5 is ubiquitously expressed in mouse tissues. *A*, PRMT5 protein levels in mouse tissues were determined by Western blot analysis. *BAT*, brown adipose tissue; *iWAT*, inguinal WAT. *B*, localization of PRMT5 in the mouse AML12 liver cell line. PRMT5 expression was visualized by immunofluorescent staining (*green*), the cytoskeleton was labeled by immunofluorescent staining of tubulin (*red*), and nuclei were stained by DAPI (*blue*). *Scale bars* = 200 μm. All images represent three independent experiments.

A high-fat diet increases hepatic PRMT5 expression

PRMT5 mRNA and protein levels were significantly increased in the livers of mice fed a high-fat diet (HFD) compared with those fed a regular diet (RD), as assessed by Western blot analysis (Fig. 2*A*), quantitative PCR (Fig. 2*B*), and immunofluorescent staining of liver sections (Fig. 2*C*). In contrast, the high-fat diet did not modify PRMT5 expression in other metabolically relevant tissues, such as muscle and brown and inguinal white adipose tissue (Fig. 2*D*). To further elucidate the role of PRMT5 in hepatic steatosis, we asked whether PRMT5 upregulation in hepatocytes is associated with obesity by examining PRMT5 levels in the livers of Agouti mice. Yellow Agouti mice with dominant mutations at the Agouti locus develop obesity, hyperinsulinemia, insulin resistance, hyperglycemia, hyperleptinemia, and hepatic steatosis in adulthood (31–33). Young Agouti mice are lean, but adult animals are obese even when they are fed regular chow (32). Therefore, the obesity in adult Agouti mice is independent of a high-fat diet. Unlike the diet-induced obesity model, PRMT5 protein levels were not different in the livers of obese 5-month-old *versus* normal weight 1-month-old Agouti mice (Fig. 2*E*). This suggested that the increase of PRMT5 was not associated with obesity *per se* but with high-fat diet–induced obesity.

PRMT5 gene silencing decreases PI3K/AKT signaling

We sought to dissect the molecular mechanism underlying the regulation of hepatic lipid homeostasis by PRMT5. Because PRMT5 is overexpressed in many cancer cell lines (29, 34–36), we generated PRMT5 knockdown in the nontransformed mouse liver cell line AML12 to avoid confounding metabolic
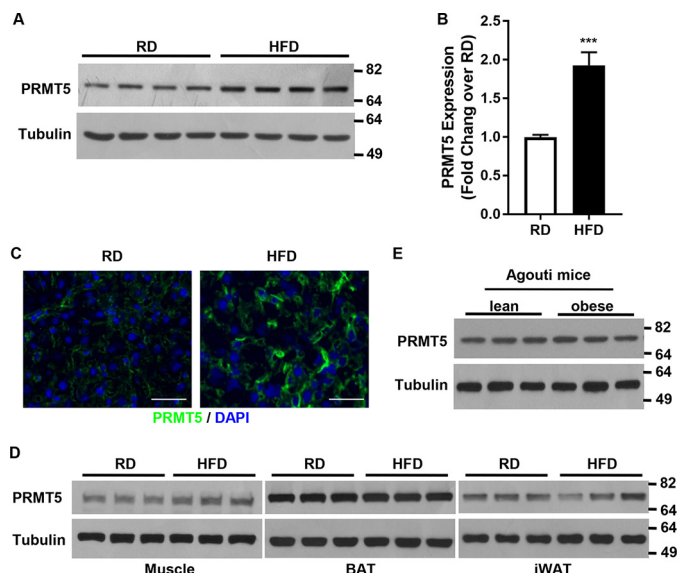
Figure 2. A high-fat diet increases hepatic PRMT5 expression. *A* and *B*, PRMT5 protein (*A*) and mRNA (*B*) levels in livers of mice fed RD or HFD were examined by Western blot analysis or qRT-PCR. Total protein or RNA was extracted from livers of 4-month-old male mice after 12 weeks of feeding with RD or HFD, respectively. *C*, PRMT5 expression in liver sections of the above samples were analyzed by immunofluorescent staining of PRMT5 (*green*) and DAPI nuclear staining (*blue*). *Scale bars* = 200 μm. *D*, PRMT5 levels in muscle, brown, or white adipose tissues from a normal diet or high-fat diet were analyzed by Western blotting. *BAT*, brown adipose tissue; *iWAT*, inguinal WAT. *E*, PRMT5 expression in the livers of lean or obese Agouti mice was measured by Western blotting. Total protein was extracted from livers of RD-fed 1- or 5-month-old Agouti mice. ***, $p < 0.001$, two-tailed Student's *t* test.

effects of cancer-related factors in transformed cell lines. As shown in Fig. 3*A*, reduction of PRMT5 expression was achieved by shRNA-mediated gene silencing. A global signaling network analysis measuring 70 key proteins involved in more than 20 signaling pathways showed that PRMT5 knockdown decreased the PI3K–AKT pathway (Fig. 3*B*). The reduction of phospho-AKT Ser-473 was detected in the array (Fig. S1), which was confirmed by a Western blot analysis that also showed decreased Thr-308 phosphorylation on AKT in PRMT5 knockdown cells (Fig. 3*C*). To confirm this finding, we utilized the inducible PRMT5 knockout mouse embryonic fibroblast (MEF) cell line, which harbors an estrogen receptor (ER)-Cre directed PRMT5$^{flox/flox}$ allele (37). When treated with 4-hydroxytamoxifen (4-OHT), the Cre recombinase translocates to the nucleus and excises an exon of PRMT5 to disrupt its expression. The PRMT5$^{flox/flox}$ MEF cells were cultured in the presence of 2 μM 4-OHT for 10 days, followed by DMEM for 3 days without 4-OHT. The efficiency of PRMT5 knockout was confirmed by Western blot analysis (Fig. 3*D*). In this PRMT5 knockout model, complete loss of PRMT5 led to a decrease in phospho-AKT Thr-308 and Ser-473. In contrast, up-regulation of phospho-AKT Ser-473 and Thr-308 was detected in the livers of mice fed a high-fat diet (Fig. 3*E*).

PRMT5 regulates the expression of master transcription factors of mitochondrial biogenesis

PPARα has a central role in fatty acid β-oxidation, lipid and lipoprotein metabolism, the inflammatory response, and oxidative stress in the liver. Activation of PPARα induces expression of mitochondrial genes in the oxidation pathways (38). Peroxi-

some proliferator–activated receptor γ, coactivator 1α (PGC-1α) is a transcriptional co-activator that regulates the expression of genes involved in energy metabolism. It is the master regulator of mitochondrial biogenesis (39–41). We surveyed the expression of these transcription factors in PRMT5 knockdown cells. Silencing PRMT5 significantly increased the expression of PPARα and PGC-1α (Fig. 4, *A* and *B*). These results were confirmed in PRMT5 knockout MEF cells (Fig. 4*C*). Consistent with increased expression of PPARα and PGC-1α, the expression of mitochondrial biogenesis genes such as CytoC and Cox7a-1 was elevated in PRMT5 knockdown cells (Fig. 4*D*). These results were replicated in PRMT5 knockout MEF cells, where expression of these genes was up-regulated (Fig. 4*E*) and mitochondrial staining was significantly increased (Fig. 4*F*). Activation of PI3K/AKT signaling has been shown to induce the expression of SREBP1(42, 43). Liu *et al.* (29) showed that, in the liver cancer cell line HepG2, silencing PRMT5 decreased SREBP1a translation but not transcription. We examined, in PRMT5 knockdown cells, the expression of acetyl-CoA carboxylase and FASN, the direct downstream targets of SREBP1 in *de novo* fatty acid synthesis. The levels of these proteins were not affected upon silencing PRMT5 (Fig. S2), which suggested that *de novo* fatty acid synthesis is not perturbed by PRMT5 silencing. It has been shown that insulin regulates lipid deposition in the goose liver by activating PI3K/AKT/mTOR signaling, which suppresses VLDL assembly and secretion (44). VLDL assembly involves transfer of lipids by the microsomal triglyceride transfer protein (MTP) to ApoB and fusion of ApoB-containing precursor particles with triglyceride droplets to form mature VLDL (45). Although PRMT5 knockdown in mouse liver cells reduced PI3K/AKT signaling, it did not affect phospho-mTOR or ApoB and MTP gene expression (Fig. S2). In contrast, in PRMT5 knockout MEF cells, loss of PRMT5 significantly decreased the levels of phospho-AKT but also had no effect on the expression of phospho-mTOR (Fig. S3).

PRMT5 enzymatic activity is required for the regulation of PGC-1α and PPARα expression

Next we asked whether the enzymatic activity of PRMT5 is required for its regulation of PGC-1α and PPARα expression. To address this question, a WT PRMT5 or a catalytically dead PRMT5 (G367A,R368A) double mutant (46) was introduced into AML12 cells by transient transfection (Fig. 5*A*). As opposed to the enzyme-dead mutant, the expression of exogenous WT PRMT5 elevated phospho-AKT Thr-308 and Ser-473 and markedly reduced PPARα (Fig. 5*B*). EPZ015666 (GSK3235025) is a potent and selective small-molecule inhibitor targeting the enzymatic activity of PRMT5 with an IC$_{50}$ of 22 nM in biochemical assays (47). Although EPZ015666 decreases cell proliferation in various cancer lines (48, 49), it did not inhibit proliferation in AML12 cells, not even in the presence of high-dose EPZ015666 for 8 weeks (Fig. S4). On the contrary, EPZ015666 treatment markedly decreased phospho-AKT Thr-308 and Ser-473 but increased PPARα in a dose-dependent manner (Fig. 5*C*). The expression of PPARα and PGC-1α, the master regulators of mitochondrial biogenesis, was elevated in the presence of EPZ015666 in a dose-dependent
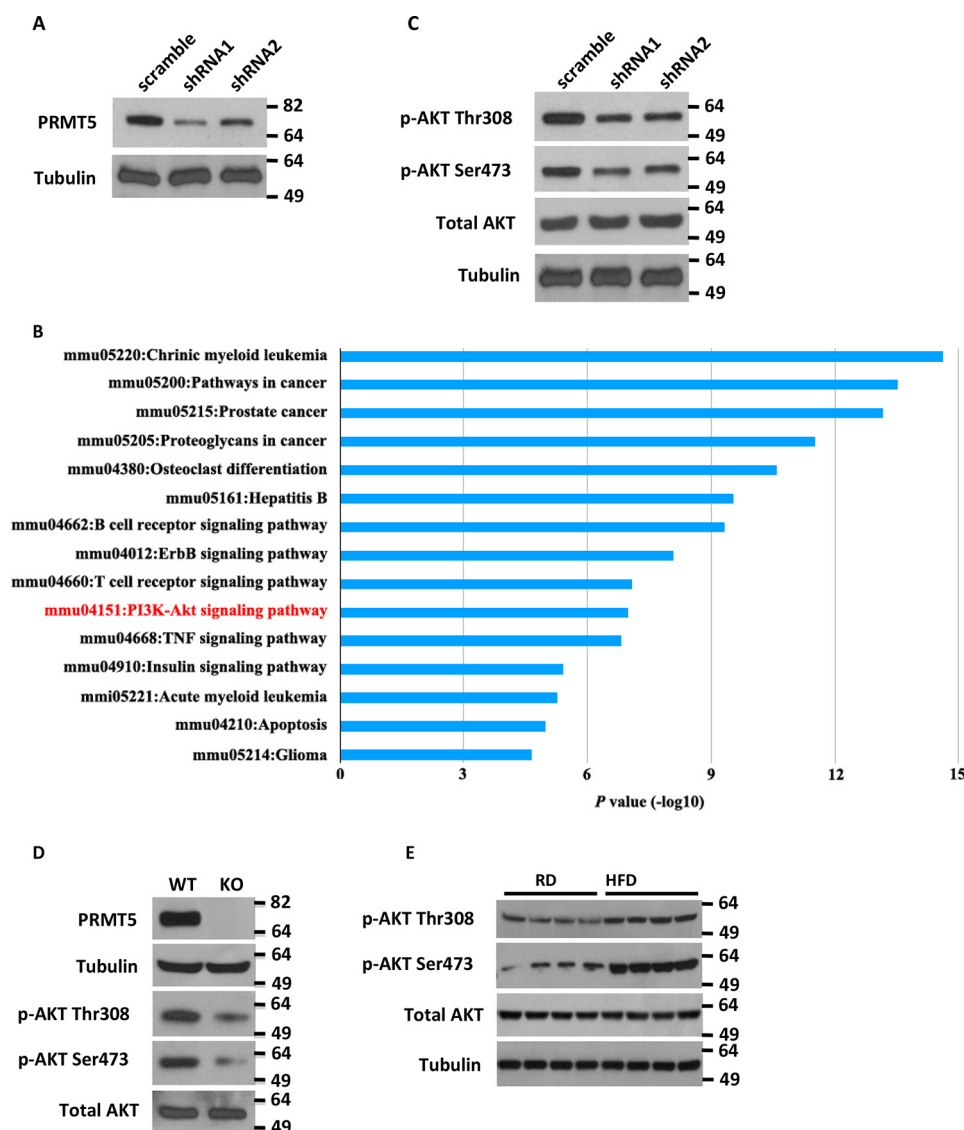
Figure 3. Silencing PRMT5 decreases PI3K/AKT signaling. *A*, Western blot analysis of PRMT5 in AML12 cells following lentiviral shRNA knockdown of PRMT5. *B*, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed proteins with PRMT5 knockdown. Total protein extracted from scramble or PRMT5 knockdown cells was analyzed for the expression of key proteins involved in more than 20 signaling pathways. Signals were normalized to α- and β-tubulin. Results were obtained from two independent experiments performed in duplicate. *C* and *D*, reduction of phospho-AKT Thr-308 and Ser-473 was observed in PRMT5 knockdown liver cells (*C*) as well as PRMT5 knockout MEF cells (*D*). *KO*, knockout. *E*, a high-fat diet up-regulated the hepatic expression of phospho-AKT Thr-308 and Ser-473. Total protein was extracted from livers of 4-month-old male mice fed either a normal or high-fat diet for 12 weeks.

manner (Fig. 5*D*) with concomitantly increased density in mitochondrial staining (Fig. 5*E*). Inhibition of PRMT5 enzymatic activity did not change the phosphor-mTOR levels in AML12 cells (Fig. S5). As a biological consequence of increasing mitochondrial biogenesis and promoting fatty acid β-oxidation, lipid accumulation in cells treated with the PRMT5 inhibitor was significantly reduced (Fig. 5*F*). Collectively, these results suggest that the enzymatic activity of PRMT5 is required to execute its function in metabolic pathways.

PRMT5 regulates PGC-1α and PPARα through phospho-AKT signaling

We sought to dissect whether PRMT5 directly regulates the expression of PPARα and PGC-1α or indirectly mediates this through phospho-AKT signaling. By analyzing the published PRMT5 ChIP-seq data (GEO: GSE75739) (50), we failed to

identify significant PRMT5 binding above the IgG control at these gene loci (Fig. S6). Introducing an exogenous activated AKT (Myr-AKT) to PRMT5 WT or knockout cells attenuated the increased expression of PPARα and PGC-1α because of the loss of PRMT5 (Fig. 6*A*). This observation was confirmed in AML12 hepatocytes, where the up-regulation of PPARα and PGC-1α in PRMT5 knockdown cells was blocked by overexpression of Myr-AKT (Fig. 6*B*). These results suggested that the regulation of PPARα and PGC-1α by PRMT5 was mediated through phospho-AKT signaling, although we could not completely rule out the possibility that PRMT5 might directly regulate these genes.

Discussion

In this study we showed that high-fat diet–induced obese mice have increased levels of PRMT5 in the liver but not in
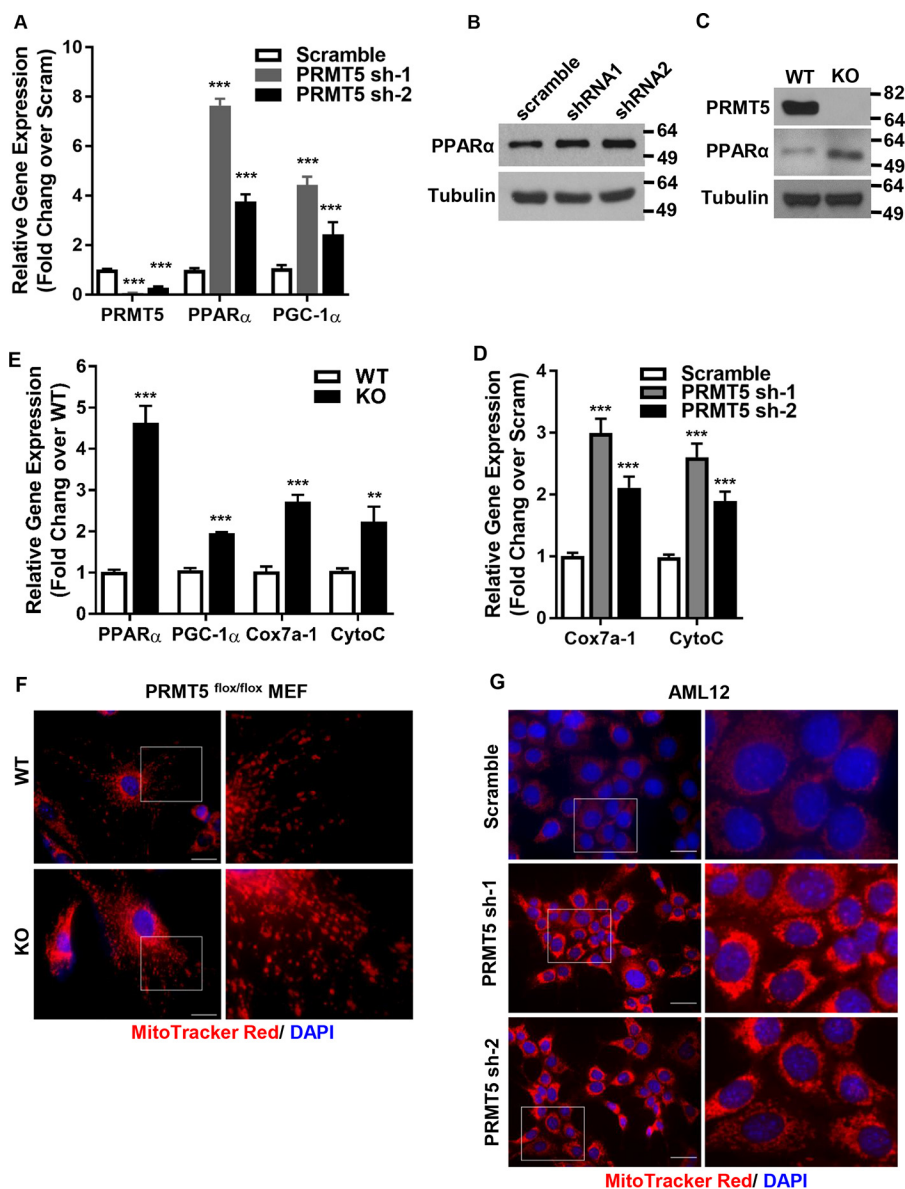
Figure 4. PRMT5 regulates master transcription factors of fatty acid metabolism and mitochondrial biogenesis. *A*, qRT-PCR analysis of the mRNA levels of PRMT5, PPARα, and PGC-1α in AML12 cells expressing either a scrambled sequence shRNA or shRNA-PRMT5. *B*, Western blot analysis of PPARα in scramble and shRNA-PRMT5 AML12 cells. *C*, Western blot analysis of PRMT5 and PPARα in WT MEF and PRMT5 knockout MEF cells. *KO*, knockout. *D*, expression of mitochondrial biosynthesis genes by qRT-PCR in scramble or shRNA-PRMT5 cells. *E*, relative mRNA expression of mitochondrial biosynthesis genes measured by qRT-PCR in WT MEF and PRMT5 knockout MEF cells. *F* and *G*, mitochondria in PRMT5^flox/flox WT or knockout MEF (*F*) and AML12 cells with scramble or PRMT5 knockdown (*G*) were stained with the mitochondrion-selective dye MitoTracker™ Red CMXRos and nuclei were labeled with DAPI (*blue*). *Scale bars* = 200 μm. Each *column* represents the mean of three independent experiments performed in triplicate. *Error bars* represent standard deviations. **, $p < 0.01$; ***, $p < 0.001$; two-tailed Student's *t* test.

other metabolically active organs, such as adipose tissues and muscle. We found that this elevated hepatic PRMT5 is associated with a high-fat diet rather than obesity *per se*. It has been shown that HFD dramatically changed chromatin structure, transcription factor binding, and the gene expression landscape in the mouse liver. Many genes associated with lipid, steroid, cholesterol, and amino acid metabolism, gluconeogenesis, the inflammatory response, oxidative stress, and oxidoreductase activity were altered. It is not clear whether up-regulation of PRMT5 is one of the early events that further shape the chromatin environment and impact gene expression under HFD or whether it is regulated by other key transcription factors involved in this process.

PI3K/AKT signaling plays a critical role in regulating diverse cellular functions, including metabolism, growth, proliferation, cell survival, transcriptional activity, and protein synthesis. Dysregulation of the PI3K/AKT pathway is implicated in cancer, type 2 diabetes, insulin resistance, inflammatory and autoimmune disorders, and cardiovascular and neurological diseases (12). The comprehensive understanding of the AKT signaling network has been expanded with the identification of numerous downstream pathways (49–51). In contrast, our knowledge of the upstream regulator or activator of AKT is limited. Wei *et al.* (30) reported that overexpressing GFP-PRMT5 led to an increase in phospho-AKT levels in 293T cells. However, this observation was not further validated with the
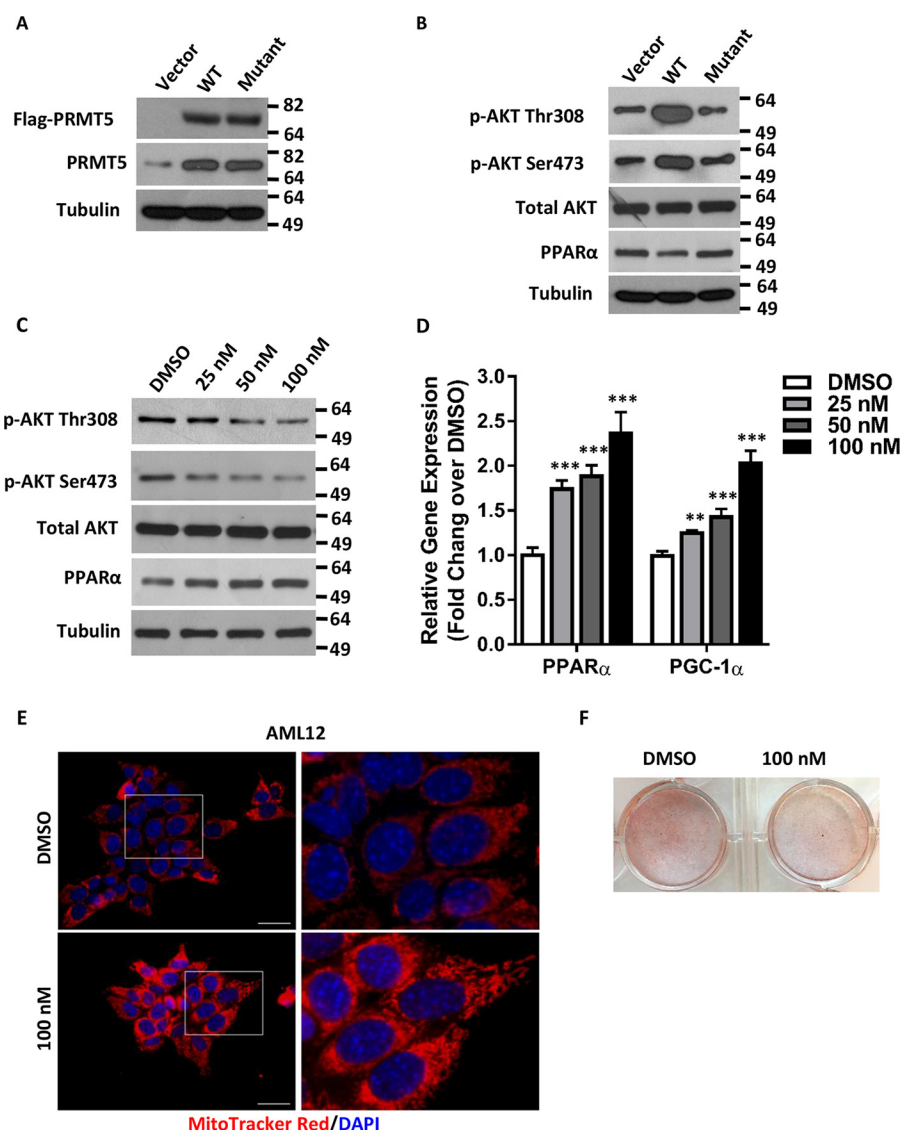
Figure 5. PRMT5 enzymatic activity is required for its regulation of PPARα expression. *A*, a FLAG-tagged WT or enzyme-dead (G367A,R368A) double PRMT5 mutant was introduced to AML12 cells by transient transfection. The expression of exogenous proteins was examined by Western blot analysis using anti-FLAG antibody. The total levels of PRMT5 were measured by Western blotting against anti-PRMT5 antibody. *B*, AKT phosphorylation and expression of PPARα in WT or mutant PRMT5-expressing cells were analyzed by Western blotting. *C*, protein levels of phospho-AKT and PPARα were determined by Western blotting in AML12 cells treated with DMSO or increasing doses of the PRMT5 inhibitor EPZ015666. *D*, the relative mRNA of PPARα and PGC-1α was measured by qRT-PCR in AML12 cells treated with DMSO or increasing doses of EPZ015666. *E*, AML12 cells treated with DMSO or 100 nM EPZ015666 were stained by MitoTracker™ Red CMXRos (*red*), and nuclei were labeled with DAPI (*blue*). *Scale bars* = 200 μm. ***, $p < 0.001$; two-tailed Student's *t* test. *F*, AML12 cells pretreated with DMSO or 100 nM EPZ015666 were exposed to medium containing 1 μM palmitic acid and 100 μM oleic acid for 5 days. Intracellular lipids were stained with Oil Red O.

endogenous protein (30). In this study, we identified endogenous PRMT5, an epigenetic modifier, as the upstream regulator of AKT activation in the context of high fat diet-induced fatty liver. Wei *et al.* (30) also showed that overexpressing GFP-PRMT5 increased mTOR Ser-2448 phosphorylation, which might be the mechanism by which PRMT5 regulates phospho-AKT. We did not detect any change in phospho-mTOR Ser-2448 levels in PRMT5 knockout MEF cells or in AML12 cells with PRM5 knockdown or treated with EPZ015666 (Figs. S2, S3, and S5). In this study, PRMT5 regulation of phospho-AKT appears to be independent of mTOR signaling. AKT activation involves the phosphorylation of two residues, Thr-308 in the activation loop and Ser-473 in the C-terminal hydrophobic motif. Phosphorylation of Ser-473 has been extensively studied

in cancers as a marker for full AKT activity (52–54). Other studies also showed that Thr-308 is predominantly phosphorylated in activated AKT kinase in tumor cells (55–57). In our study, knockdown of PRMT5 or treatment with the PRMT5 enzymatic inhibitor EPZ015666 in hepatocytes attenuated both phospho-AKT Thr-308 and Ser-473. Our results indicate that targeting PRMT5 blocks the activation of AKT, which may have a profound effect on its downstream signaling networks.

Mitochondrial dysfunction is associated with many diseases, such as type 2 diabetes and neurodegenerative diseases (58, 59). PGC-1α is a master regulator of mitochondrial biogenesis by activating the transcription factors nuclear respiratory factor 1 and nuclear respiratory factor 2. These induce mitochondrial transcription factor A, which drives the transcription and rep-
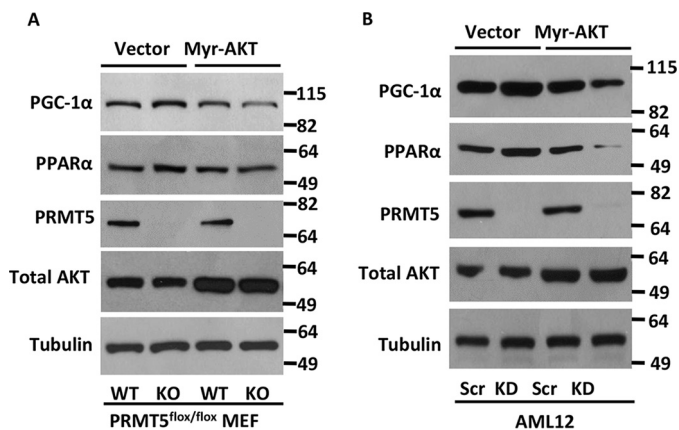
Figure 6. PRMT5 regulates PPARα and PGC-1α expression via AKT signaling. *A*, PRMT5 $^{flox/flox}$ WT or knockout MEF cells were transfected with vector or Myr-AKT and harvested 48 h post-transfection. Protein samples were analyzed by Western blotting for PPARα and PGC-1α expression. *KO*, knockout. *B*, AML12 cells were transduced with scramble or shPRMT5 viral supernatant for 24 h and then selected in medium containing 2 µg/ml puromycin for 48 h. Then cells were transfected with vector or Myr-AKT and harvested 48 h post-transfection. Protein samples were analyzed by Western blotting for PPARα and PGC-1α expression. *KD*, PRMT5 knockdown.

lication of mitochondrial DNA (59, 60). PGC-1α expression levels are regulated in response to a plethora of stimuli, such as exercise, feeding, fasting, and cold exposure, and modulated by numerous factors, such as Ca^{2+}/calmodulin– dependent protein kinase IV (CaMKIV) and calcineurin A, AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinase (p38 MAPK) (61, 62). We here report that PRMT5 repressed the expression of PGC-1α in hepatocytes. Silencing or complete loss of PRMT5 increased the expression of PGC-1α and mitochondrial biogenesis. AKT was found to phosphorylate and inhibit PGC-1α (63). PRMT5 ChIP-seq in MDA-MB-231 cells did not identify PRMT5 binding sites at the gene loci of PPARα and PGC-1α (50). With no PRMT5 ChIP-seq data from hepatocytes, we cannot completely dismiss the possibility that PRMT5 might have a direct role in regulating these genes. Introducing activated Myr-AKT to PRMT5 knockout MEF or knockdown AML12 cells decreased the up-regulation PPARα and PGC-1α. These results suggest that it is more likely that PRMT5 regulates the expression of PPARα and PGC-1α via AKT signaling.

Overexpression of PRMT5 in various cancers has been linked to cancer progression and poor prognosis (34, 64). Therefore, PRMT5 is well-recognized as a viable therapeutic target in cancer treatment, and a number of inhibitors have been developed within the past couple of years to target cancer, β-thalassemia, or sickle cell anemia. EPZ015666 is a potent PRMT5 inhibitor that has been shown to be active *in vivo* (47). A first time in human, open-label, dose escalation study of GSK3326595, a potent and selective PRMT5 inhibitor similar to EPZ015666, in subjects with advanced or recurrent solid tumors as well as subjects with a subset of solid tumors and non-Hodgkin's lymphoma was approved by the Food and Drug Administration and started in August 2016 (ClinicalTrials.gov identifier NCT02783300). We now report that inhibiting PRMT5 by EPZ015666 markedly decreased both phospho-AKT Thr-308 and phospho-AKT Ser-473, up-regulated PPARα and PGC-1α

expression, and significantly increased mitochondrial biogenesis. Excessive intake of dietary lipids was shown to decrease hepatic PPARα and PGC-1α expression with a reduced numerical density of hepatic mitochondria (65). A number of PPARα agonists have been shown to enhance hepatic mitochondrial biogenesis and increase fatty acid β-oxidation in diet-induced obese mice (66, 67). As an upstream regulator of AKT, PPARα, and PGC-1α, PRMT5 might be a valuable target to treat fatty liver via multiple mechanisms or may be even more broadly applied to other diseases that are associated with mitochondrial dysfunction. Chen *et al.* (68) reported that PRMT5 methylates GATA 4 and inhibits its transcriptional activity in cardiomyocytes, which is important in prevention of cardiac hypertrophy and heart failure. Thus, the balance of PRMT5 levels in the context of different diseases needs to be considered when developing PRMT5 inhibition– based therapies.

Experimental procedures

Animals

C57BL/6 (BL6) mice were housed in a temperature-controlled environment with a 12-h light, 12-h dark cycle and fed a standard chow *ad libitum*. Starting at 8 weeks of age, male mice were fed *ad libitum* either an RD (12% fat content) or HFD (45% fat content, catalog no. D12451, Research Diets, New Brunswick, NJ) for 3 months (69). Animals were then euthanized, and tissue samples were collected to be either snap-frozen in liquid nitrogen or embedded in optimal cutting temperature (OCT) compound blocks. The Agouti mice were housed in a temperature-controlled environment with a 12-h light, 12-h dark cycle and fed a standard chow *ad libitum*. All procedures were approved by the Institutional Animal Care and Utilization Committee at the University of Toledo.

Cell culture

The nontransformed α mouse liver 12 (AML12) cell line was provided by Dr. Anthony Imbalzano (University of Massachusetts Medical School). This cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human transforming growth factor α (70). AML12 cells retain the capacity to express high levels of serum- and liver-specific proteins (70). These cells were maintained in monolayers, as described previously (70). They were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 µg/ml streptomycin, insulin (5 µg/ml), transferrin (5 µg/ml) and sodium selenite (5 ng/ml) (ITS) (Invitrogen). Human embryonic kidney 293T cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum and 100 µg/ml streptomycin. PRMT5 $^{flox/flox}$ MEFs were immortalized PRMT5 $^{flox/flox}$ mouse embryonic fibroblasts and obtained from Dr. Mark Bedford (University of Texas MD Anderson Cancer Center).

Lentiviral shRNA construction and transduction

PRMT5 shRNA knockdown lentiviral constructs were prepared using the psp-108 vector (Addgene), and the lentivirus particles were produced by co-transfection with plasmids pMD2.G (Addgene) and psPAX2 (Addgene) into human

embryonic kidney 293T cells. Titers were determined, and the same number of virus was used, as indicated. PRMT5 knockdown targeting sequences were as follows: shRNA1, 5′GCACAGTTTGAGATGCCTTAT3′; shRNA2, 5′CCTCT-TGTGAATGCGTCTCTT3′. The FLAG-PRMT5 and FLAG-PRMT5 (G367A,R368A) double mutant were from Dr. Said Sif (Qatar University) (46).

Transient transfection

Cells were plated in 6-well plates or 60-mm dishes to 60–70% confluency and then transfected with 6 or 15 μg of plasmid DNA, respectively, using Lipofectamine 2000 transfection reagent, following the manufacturer's protocol. Cells were harvested 48 h post-transfection for protein analysis by Western blotting.

Immunofluorescence

Cells were seeded on glass coverslips and incubated overnight. For mitochondrion staining, cells were incubated with MitoTracker^{TM} Red CMXRos (250 nM, Thermo Fisher, catalog no. M7512) at 37 °C for 30 min before being fixed with 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization in ice-cold methanol for 10 min at −20 °C. Cells were then incubated in blocking buffer (5% BSA in PBS) for 1 h at room temperature, followed by incubation with anti-PRMT5 antibody (Santa Cruz Biotechnology, catalog no. sc-376937) and anti-β-tubulin antibody (Cell Signaling Technology, catalog no. 15115) at 4 °C overnight. Cells were washed three times for 5 min in PBS and then incubated for 2 h with Alexa Fluor 488–conjugated goat anti-mouse secondary antibody against PRMT5 and Alexa Fluor 555–conjugated goat anti-rabbit secondary antibody for β-tubulin (diluted 1:100 in blocking buffer, Invitrogen) at room temperature. Finally, the nuclei were stained with DAPI for 30 min at room temperature before visualization.

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized and rehydrated through graded ethanol solutions. The slides were incubated with blocking buffer (PBS containing 5% normal goat serum and 0.3% Triton X-100) for 60 min and then the slides were incubated with PRMT5 antibody at 4 °C overnight. Subsequently, the slides were washed with PBS, and incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and DAPI for 2 h before observation using a Leica fluorescence microscope.

RT-qPCR

Total RNA was extracted from AML12 cells or MEF cells using TRIzol regent (Invitrogen), and cDNA was synthesized with the SuperScript Reverse Transcriptase III kit (Invitrogen) according to the manufacturer's instructions. The cDNA was amplified in 96-well reaction plates with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 real-time PCR thermocycler. The sequences of forward and reverse primers are listed in Table S1. The relative levels of target transcripts were calculated from duplicate samples after normalization against the U36 housekeeping gene. Dissociation curve analysis was performed after PCR amplification to confirm primer spec-ificity. Relative mRNA expression was calculated using the $\Delta\Delta C_T$ method.

Western blot analysis

Tissues or cells were washed with cold PBS and lysed in 1% NP40 lysis buffer containing a protease and phosphatase inhibitor mixture (Roche Diagnostics). Proteins were analyzed by SDS-PAGE, followed by immunoprobing with the following antibodies: PPARα, sc-398394; PRMT5, sc-376937; acetyl-CoA carboxylase α, sc-137104; FASN, sc-48357; mTOR, sc-293089; and p-mTOR, sc-293133. For normalization, monoclonal antibodies against β-tubulin (Developmental Studies Hybridoma Bank, E7-S) were used. Blots were incubated with horseradish peroxidase–conjugated anti-goat IgG (Santa Cruz Biotechnology) and anti-mouse or anti-rabbit IgG (GE Healthcare) antibodies, and proteins were detected by ECL (GE Healthcare).

Fatty acid treatments

Cells were pretreated with increasing doses of EPZ015666 for 6 days, seeded at a density of 10,000 cells/well in 24-well plates, and cultured for 24 h. Palmitic acid (Sigma-Aldrich, catalog no. P0500) and oleic acid (Sigma-Aldrich, catalog no. O1008) were prepared by conjugation with nonfat BSA (Sigma-Aldrich, catalog no. A8806). Cells at 50% confluency were exposure to 1 $\mu$$M$ palmitic acid and 100 $\mu$$M$ oleic acid in the presence of EPZ015666 for 5 days. Lipid droplets were stained with Oil Red O.

Oil Red O staining

To assess intracellular neutral lipid, dietary fatty acids-treated cells were fixed using 10% formalin and stained using Oil Red O (Sigma-Aldrich) for 30 min at room temperature. Stained cells were washed with water and rinsed with 60% isopropanol. Lipid accumulation was visualized using light microscopy.

MTT assay

Cell proliferation was measured using the MTT assay. Three thousand cells from each treated group were plated in a 96-well plate. Cell proliferation was measured at 24h intervals by adding MTT solution (final concentration, 5 μg/ml) to each well and incubating for 4 h. After removal of the medium, the plate was air-dried, and 100 μl of DMSO was added. The plate was incubated at room temperature for 30 min with gentle shaking. Absorbance was measured at A_{540} in a Synergy H4 Hybrid microplate reader (Bio Tek, Winooski, VT).

ActivSignal IPAD assay

Cells were lysed in PBS + 1% NP40 lysis buffer and submitted to ActivSignal for further processing. The expression or phosphorylation of 70 key proteins involved in more than 20 signaling pathways was analyzed by antibodies with high specificity and sensitivity in combination with two distinct antibodies per target. The signal was normalized to the expression of housekeeping genes. Each pathway was covered by multiple targets. The experiment was performed twice.

KEGG pathway enrichment analysis

Proteins with more than a 1.5-fold change after PRMT5 knockdown in immuno-paired–antibody detection (IPAD) assay were fetched to perform pathway enrichment analysis using DAVID (71). Pathway annotations from the KEGG pathway database with a p value smaller than 0.05 were defined as significantly enriched (72).

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