Pyruvate kinase M2 interacts with nuclear sterol regulatory element-binding protein 1a and thereby activates liprogenesis and cell proliferation in hepatocellular carcinoma

Xiaoping Zhao†‡, Li Zhao†*, Hao Yang§, Jiajin Li†, Xuejie Min‡, Fajun Yang§†, Jianjun Liu†, Gang Huang†§‡

† Department of Nuclear Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China.
§ Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China.
‡ Department of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, NY 10461, USA.

* These authors contributed equally to this work.
† To whom correspondence may be addressed: F.Y. (fajun.yang@einstein.yu.edu), X.Z. (zxp0856@sina.com), J.L. (nuclearj@163.com), or G.H. (huang2802@163.com)

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Dysregulation of lipid metabolism is common in cancer cells, but the underlying mechanisms are poorly understood. Sterol regulatory element–binding proteins (SREBPs) stimulate lipid biosynthesis through transcriptional activation of lipogenic enzymes. However, SREBPs’ roles and potential interacting partners in cancer cells are not fully defined. Using a biochemical approach, we found here that pyruvate kinase M2 (PKM2) physically interacts with the nuclear form of SREBP-1a (nBP1a), by binding to amino acids 43–56 in nBP1a. We also found that PKM2 activates SREBP target gene expression and lipid biosynthesis by stabilizing nBP1a proteins. Using a competitive peptide inhibitor to block the formation of the SREBP-1a/PKM2 complex, we observed that this blockade inhibited lipogenic gene expression. Of note, nBP1a phosphorylation at Thr-59 enhanced the binding to PKM2 and promoted cancer cell growth. Moreover, we show that PKM2 phosphorylates Thr-59 in vitro. Lastly, in human patients with hepatocellular carcinoma, nBP1a phosphorylation at Thr-59 was negatively correlated with clinical outcomes. Together, our results reveal that nBP1a/PKM2 interaction activates lipid metabolism genes in cancer cells and that Thr-59 phosphorylation of SREBP-1a plays an important role in cancer cell proliferation.

Lipids are essential for cell proliferation. In fact, it has been estimated that more than 90% fatty acids in tumors cells are derived from de novo biosynthesis, while normal cells obtain lipids primarily from the circulation (1). In several types of cancer, including breast and prostate cancer, fatty acid synthase (Fasn) gene is up regulated (2), suggesting that fatty acid biosynthesis may play a role in cancer pathogenesis. Sterol regulatory element-binding proteins (SREBPs) are conserved transcription factors that can activate the transcription of genes encoding the key lipogenic enzymes, including Fasn (3,4). In mammalian cells, there are three SREBP isoforms (SREBP-1a, -1c and -2) encoded by two different genes, Srebf1 and Srebf2 (5). Two distinct promoters generate SREBP-1a and SREBP-1c isoforms from the Srebf1 gene (5). Interestingly, Srebf1a is expressed at higher levels in cancer cells as compared to normal tissues, while Srebf1c is the predominant product of Srebf1 in normal tissues (6). In addition, SREBP-1a is a potent transcriptional activator for all known SREBP-target genes (7). The SREBP1 protein levels are often correlated with tumor size, histological grade and metastasis, and SREBP1 loss of function inhibits cell proliferation and induces apoptosis, cell migration and invasion in liver, ovarian and endometrial cancers (8-11). Furthermore, genetic depletion or pharmacological inhibition of SREBP1 has been
shown to suppress the epidermal growth factor receptor (EGFR)-induced glioblastoma (12). Blocking SREBP pathway prevents hepatocellular carcinoma (HCC) in mouse models (13). Thus, SREBP1 is required to support proliferation in some cancer cells.

Previous studies have shown that pyruvate kinase isozyme M2 (PKM2) plays an important role in the Warburg effect (14). PKM2 and its isoform PKM1 are products of the Pkm gene through alternative splicing (15). It has been reported that in most tumors the Pkm2 mRNA is elevated (16,17) and in some cases the Pkm gene expression is switched from Pkm1 to Pkm2 (17). Several studies have revealed that PKM2 can translocate into nucleus and acts as a transcriptional cofactor to promote tumor development (18-20).

Here, we identified a novel SREBP-1a/PKM2 protein complex. We show that PKM2 stimulates SREBP1-dependent cancer cell proliferation, and activates SREBP-target gene expression by stabilizing nuclear SREBP-1a proteins.

Results

Nuclear SREBP-1a interacts with PKM2 in cancer cells

Protein-protein interaction is one of the key regulatory mechanisms in biology. To better understand the regulation of SREBP-1a in the nucleus, we overexpressed a Flag-tagged nuclear form of SREBP-1a (Flag-nBP1a) in HEK293T cells and screened for novel SREBP1a-binding proteins by co-immunoprecipitation (Co-IP) of nuclear extracts (Fig. 1A). Among the eluted proteins following IP with anti-Flag antibody, we detected multiple peptides of PKM2 proteins by liquid chromatography-tandem mass spectrometry (data not shown), indicating that PKM2 binds to Flag-nBP1a. Multiple lines of evidence independently confirmed the interaction between SREBP-1a and PKM2. By immunoblotting of IP samples, we found that overexpressed Flag-nBP1a could pull down endogenous PKM2 (Fig. 1B) and overexpressed wild-type PKM2 (HA-PKM2-WT) (Fig. 1C). Immunostaining analyses revealed that Flag-nBP1a proteins were co-localized with HA-PKM2-WT in the nuclei, whereas Flag-nBP1a and cytoplasmic-localized K367M mutant PKM2 proteins (HA-PKM-K367M) (19) were rarely co-localized (Fig. 1D).

Moreover, although SREBP-1a and SREBP-1c differ only at the very N-terminal ends (Fig. 1E), PKM2 only interacted with SREBP-1a, but not SREBP-1c or SREBP-2 (Fig. 1F). Conversely, both overexpressed HA-PKM2-WT (Fig. 1G) and endogenous PKM2 (Fig. 1H) could pull down endogenous nuclear form of SREBP1 proteins, which are presumably the SREBP-1a isoform because of the higher expression of its mRNA (21). To determine whether PKM2 directly interacts with SREBP-1a, we prepared GST fusion proteins of PKM2 and 6xHis-tagged nuclear form of SREBP-1a. As shown in Fig. 1I, recombinant PKM2 could also bind to recombinant SREBP-1a in GST pull-down assays. Thus, our data demonstrates that PKM2 directly interacts with the nuclear form of SREBP-1a in cancer cells.

PKM2 regulates nuclear SREBP-1 protein stability

To understand the significance of PKM2 binding to SREBP-1a in cancer cells, we analyzed PKM2 and SREBP1 proteins (likely including both SREBP-1a and SREBP-1c) in tumor samples from patients with HCC by immunohistochemistry (Fig. 2A). Semi-quantitative analyses of samples from 75 patients revealed that the protein levels of SREBP1 were significantly correlated with those of PKM2 in human HCC (Fig. 2B). This data suggests that PKM2 may increase SREBP1 protein stability in HCC cells, because nuclear SREBP1 proteins are normally unstable. To examine this possibility, we knocked down PKM2 in HepG2 cells by PKM2-specific siRNA. Non-specific (NS) siRNA was used as control. Indeed, PKM2 knockdown significantly reduced the protein levels of nuclear SREBP1, while the precursor form of SREBP1 was essentially not affected (Fig. 2C). Moreover, PKM2 knockdown did not change the mRNA levels of Srebf1 gene in HepG2 cells (data not shown). This data indicates that PKM2 positively regulates nuclear SREBP1 protein levels.

To determine whether PKM2 increases nuclear SREBP1 protein stability, we overexpressed Flag-nBP1a in HepG2 cells and treated with cycloheximide (CHX), a protein synthesis inhibitor, for various periods of time with or without PKM2 knockdown. As shown in Fig. 2D and 2E, PKM2 knockdown accelerated nBP1a degradation. It has been shown that nuclear SREBP1 proteins are degraded by proteasomes (22). Therefore, we
examined whether inhibition of proteasomes could abolish PKM2 regulation of nBP1α. For that purpose, we overexpressed Flag-nBP1α in HepG2 cells and then treated with MG132, a proteasome inhibitor, with or without PKM2 knockdown. As shown in Fig. 2F, PKM2 knockdown–caused reduction of nBP1α was blocked in the presence of MG132, suggesting that PKM2 inhibits proteasome-mediated degradation of nuclear SREBP-1α.

**T59 phosphorylation of nuclear SREBP-1α enhances its protein stability and binding to PKM2**

Since serine or threonine phosphorylation regulates nuclear SREBP1 protein stability (22,23), we wondered whether PKM2 regulates nBP1α through phosphorylation. To this end, we immunoprecipitated endogenous SREBP1 from HepG2 cells that were treated with PKM2-specific or control (NS) siRNA, and analyzed serine or threonine phosphorylation using anti-phospho-serine (p-Ser) or anti-phospho-threonine (p-Thr) antibody. As shown in Fig. 3A, when compared in a similar amount of SREBP1 proteins, while PKM2 knockdown in HepG2 cells did not affect serine phosphorylation of nuclear SREBP1, it reduced threonine phosphorylation. Together with the data in Fig. 2C, PKM2 positively controls the protein levels and threonine phosphorylation of nuclear SREBP-1α. Therefore, it is less likely that PKM2 regulates phosphorylation of the previously identified Threonine-426 (T426) residue of nBP1α (22,23), as reducing T426 phosphorylation would increase nBP1α protein levels.

To identify the novel threonine residue(s) in nBP1α whose phosphorylation is regulated by binding to PKM2, we decided to identify which domain(s) of nBP1α are responsible for interacting with PKM2. For that purpose, we generated three truncated forms of nBP1α as GST fusion proteins (Fig. 3B) and examined their ability to interact with PKM2 by GST pull-down assays. As shown in Fig. 3B, when incubated with recombinant Flag-tagged PKM2 proteins followed by extensive wash, full-length and the amino acids (aa) 1-209, but not aa61-273 or aa323-490, fragment of nBP1α could pull down PKM2. This data suggests that the aa1-60 region of SREBP-1α is required for interaction with PKM2.

There are two threonine residues (T27 and T59) within the aa1-60 region of SREBP-1α. To determine whether T27 and/or T59 phosphorylation affects nBP1α protein stability and binding to PKM2, we generated phosphor-defective mutant (T27A or T59A) of nBP1α. As shown in Fig. 3C, T59A, but not T27A, mutant of Flag-nBP1α was expressed at a level significantly lower than wild-type (WT) in HepG2 cells, indicating that T59 phosphorylation is responsible for enhancing the nBP1α protein stability. Moreover, T59A mutation not only decreased nBP1α protein levels, but also abolished PKM2 knockdown–induced reduction of nBP1α proteins (Fig. 3D). To compare the phosphorylation levels, we normalized the immunoprecipitated Flag-nBP1α proteins to the same level among different samples before immunoblotting. As shown in Fig. 3E, T59A mutation reduced threonine phosphorylation in nBP1α, and abolished PKM2 knockdown–caused reduction of threonine phosphorylation (Fig. 3E).

Since ubiquitination controls nuclear SREBP1 protein degradation (22), we examined the effects of PKM2 knockdown on ubiquitination of Flag-nBP1α. To examine the short-lived polyubiquitinated Flag-nBP1α, we co-expressed HA-tagged ubiquitin (HA-Ub) with Flag-nBP1α in HepG2 cells followed by MG132 treatment. The ubiquitination levels of immunoprecipitated Flag-nBP1α proteins were analyzed by immunoblotting using anti-HA antibody. As shown in Fig. 3F, PKM2 knockdown significantly increased nBP1α ubiquitination, correlated with the reduced levels of nBP1α proteins (Fig. 3C). Consistent with the data in Fig. 3C, T59A, but not T27A, mutation also increased nBP1α ubiquitination, while PKM2 knockdown did not result in any further increase of nBP1α ubiquitination (Fig. 3F), suggesting that PKM2-regulated T59 phosphorylation blocks ubiquitination and thus increase the stability of nBP1α proteins. To further examine this possibility, we transfected HepG2 cells with Flag-nBP1α (WT or T59A) after PKM2 knockdown, and then treated cells with CHX for various periods of time, similar to Fig. 2D. Semiquantitative analyses of immunoblotting data indicate that PKM2 knockdown or T59A mutation similarly accelerated the degradation of nBP1α (Fig. 3G). Together, these results indicate that T59 phosphorylation is a novel mechanism for enhancing the nBP1α protein stability.

PKM2 has been reported as a threonine-serine kinase of proteins, including histone H3 (24) and MLC2 (25). Thus, we examined whether PKM2
could directly phosphorylate SREBP-1a. For that purpose, we performed in vitro kinase assays using Flag-tagged PKM2 proteins that were overexpressed and immune-purified from HEK293T cell lysates as kinase and recombinant GST-nBP1a proteins as substrate (24). We detected phospho-threonine signals in recombinant SREBP-1a only when the PKM2 substrate phosphoenolpyruvate (PEP) was added to the reactions (Fig. 3H). Importantly, wild-type PKM2 become unable to phosphorylate SREBP-1a when T59 is mutated to alanine (Fig. 3I), suggesting that T59 contributes to the phosphorylation signals. Moreover, PKM2 with K367M mutation, which is kinase-dead according to the previous studies (19), is unable to increase threonine-phosphorylation in SREBP-1a. Together, our data suggests that PKM2 is able to directly phosphorylate SREBP-1a at T59.

**T59 phosphorylation of nuclear SREBP-1a is correlated with poor prognosis in HCC patients**

To determine the clinical significance of nBP1a phosphorylation at the T59 residue, we generated an antibody against T59 phosphorylated SREBP1 (p-T59-BP1). The specificity of this antibody for immunohistochemistry was first validated in HepG2 cells with overexpression of Flag-nBP1a-WT or T59A mutant, which serves as a negative control (Fig. 4A). Then, we performed phosphopeptide competition analyses in immunohistochemistry assays of HepG2 cells (data not shown) and liver samples from patients with HCC (Fig. 4B) to further confirm its specificity. Using this antibody, we examined the p-T59-BP1 levels in both tumors and the adjacent normal tissues from a total of 90 patients with HCC by immunohistochemistry. Overall, the p-T59-BP1 signals were detected mainly in the nuclei, and tumor samples displayed significantly stronger p-T59-BP1 signals than adjacent normal liver tissues (Fig. 4C), indicating that T59 phosphorylation occurs mainly in nuclear SREBP1 and T59 phosphorylation of SREBP1 is increased in human HCC. Using a scale of 0 to 7 (in which 0 to 3 stands for low and 4 to 7 for high p-T59-BP1 signals) to quantify SREBP1 phosphorylation, we found that the median survival duration was 41.9 and 28.1 months for patients with low and high scores of p-T59-BP1, respectively (Fig. 4D). The Kaplan-Meier survival curve showed that the p-T59-BP1 levels were negatively and significantly correlated with the survival duration of HCC patients (Fig. 4D). Moreover, the p-T59-BP1 levels were significantly higher in tumor tissues than in adjacent normal tissues from this group of patients with HCC (Fig. 4E). By multivariate statistical analyses, we found that the p-T59-BP1 levels were significantly correlated with histologic grade (p=0.008) and TNM stage (p=0.030) (Table 1, Fig. 4F and 4G). Using a Cox multivariate model, we found that the p-T59-BP1 level was an independent predictor of HCC patient survival (HR=2.166) (95% CI: 1.262-3.567). Together, our results revealed a good correlation between T59 phosphorylation of nuclear SREBP1 proteins and the clinical outcomes of patients with HCC.

**T59 phosphorylation of nuclear SREBP-1a is critical for cancer cell proliferation**

Consistent with the oncogenic property of PKM2, overexpression of PKM2 in HepG2 cells decreased the cell doubling time, indicating an increase of cell proliferation (Fig. 5A). However, when SREBP1 was depleted by specific siRNA, PKM2 overexpression was no longer able to enhance HepG2 cell proliferation (Fig. 5A), suggesting that SREBP1 is required for PKM2-induced cell proliferation. The SREBP1-dependency for PKM2-enhanced cell proliferation may not be limited to HCC, as we obtained similar results in SW480, A549 and MCF7 cells (Fig. 5A). To further determine the requirement of SREBP1 for PKM2-induced cell proliferation, we treated HepG2 cells with chemical SREBP inhibitors, 25-hydroxyl cholesterol (25-HC), betulin or fatostatin. As shown in Fig. 5B, in the presence of these SREBP inhibitors, PKM2 overexpression was unable to promote HepG2 cell growth.

To further study the function of SREBP1, we generated a HepG2 cell-line of SREBP1-knockout (BP1-KO) using the CRISPR/Cas9 technology (Fig. 5C). To determine the role of PKM2 regulating nBP1a in cell proliferation, we transfected HepG2 cells with WT, T27A, T59A or T27A/T59A double mutant (DM) of Flag-nBP1a (vector as transfection control). As shown in Fig. 5D, overexpression of either wild-type or T27A mutant nBP1a significantly increased cell growth as evidenced by the reduced cell doubling time. In contrast, overexpression of T59A single-mutant or T59A/T27A-DM nBP1a inhibited the growth of HeG2 cells (Fig. 5D).

To determine the role of PKM2 binding to nBP1a in tumor development in vivo, we established mouse
xenograft models by subcutaneously injecting nude mice with stable BP1-KO HepG2 cells that express Flag-PKM2 (WT) or Flag-nBP1a (WT or T59A). Stable transfection with the empty vector served as a control. As shown in Fig. 5E, when compared to vector alone PKM2 overexpression on the SREBP1-knockout background did not alter the rate of tumor growth, and thus resulted in tumors with weights similar to those from vector control (Fig. 5F). This data is consistent with that from cell culture (Fig. 5A), and indicates that PKM2 regulation of cell proliferation is SREBP1-dependent in vivo. Moreover, overexpression of nBP1a-WT on the SREBP1-knockout background significantly accelerated tumor growth (Fig. 5E) and gave rise to larger tumors than vector control (Fig. 5F), suggesting a positive role of SREBP-1a on HepG2 cell proliferation in vivo. In contrast, tumors originated from nBP1a-T59A expressing cells grew with slower rates (Fig. 5E) and gave rise to smaller tumors (Fig. 5F) than those from nBP1a-WT expressing cells, consistent with the cell culture data (Fig. 5D). Moreover, PKM2 knockdown in HepG2 cells by specific siRNA increased the cell doubling time, indicating reduced cell proliferation (Fig. 5G). Together, our in vitro and in vivo results suggest that T59 phosphorylation of SREBP-1a is required for HCC cancer cell proliferation.

PKM2 activates lipogenic gene expression and lipid biosynthesis

The well-defined SREBP-1a target genes include those encoding enzymes in both fatty acids and cholesterol biosynthesis pathways (3,4). Since PKM2 regulates the abundance of nuclear SREBP-1a (Fig. 2C), we examined whether PKM2 regulates SREBP-1a-mediated transcription. First, we analyzed the mRNA levels of SREBP-1a target genes after PKM2 knockdown. We used three different PKM2-siRNAs to exclude potential off-target effects, and the PKM2 knockdown was efficient (Fig. 2C and 6A). As shown in Fig. 6A, PKM2 knockdown significantly reduced the mRNA levels of previously defined SREBP-1a target genes, including Acc1, Acly, Elovl6, Fasn, Scd1, Hmgcr and Ldlr, in HepG2 cells, and the change of gene expression was correlated with the PKM2 knockdown efficiency. By luciferase reporter assays, we found that PKM2 knockdown significantly reduced the activity of human Fasn promoter (Fig. 6B), which has a size of 175bp, but includes all SREBP1-binding sequences. Consistent with the decrease of lipogenic gene expression, PKM2 knockdown caused a significant reduction of lipid levels in HepG2 cells as assayed by both lipid staining (Fig. 6C) and quantitative measurements of cellular triglycerides (Fig. 6D) as well as cholesterol (Fig. 6E). To further determine the effect of PKM2 knockdown on lipid levels, we quantitatively analyzed fatty acid profiles. Consistent with the gene expression data, the amount of some fatty acids, including C16:0, C18:0 and C18:1, was significantly reduced after PKM2 knockdown (Fig. 6F).

To further understand the PKM2 regulation of lipogenic gene expression, we overexpressed PKM2 in HepG2 cells and observed a significant up-regulation of SREBP-1a target genes (Fig. 6G). However, when SREBP-1 was depleted by specific siRNA, PKM2-enhanced lipogenic gene expression was compromised for some of those genes, including Acly, Elovl6, Gpat and Hmgcr (Fig. 6G). Interestingly, PKM2 could still stimulate the expression of some genes, including Fasn, from lower backgrounds that were caused by SREBP-1 depletion (Fig. 6G). To determine whether PKM2 regulation of SREBP-1a target genes is common in cancer cells, we also examined SW480, A549 and MCF-7 cells by qRT-PCR. As shown in Fig. 6G, we obtained similar results from different cell-lines, indicating that PKM2 is a novel activator for lipogenic gene expression in cancer cells. Furthermore, PKM2 overexpression also stimulated the promoter activity of Fasn in those cancer cells (Fig. 6H). However, different from the regulation of endogenous Fasn (Fig. 6G), PKM2 overexpression was unable to activate the short Fasn promoter when SREBP-1 was depleted (Fig. 6H), suggesting that PKM2 regulates endogenous Fasn gene likely through both SREBP1-dependent and -independent mechanisms. Nevertheless, our results reveal a novel function of PKM2 in regulating lipogenic gene expression and lipid biosynthesis in cancer cells.

To determine the role of T59 phosphorylation in regulating SREBP-1a target gene expression, we overexpressed Flag-nBP1a (WT, T59A or T59E) in SREBP-1 knockout HepG2 cells (Fig. 5C) and examined the mRNA levels by qRT-PCR. When compared to WT, phosphor-defective T59A mutant was less potent, while phosphor-mimic T59E mutant was more potent, to activate SREBP-1a target genes (Fig. 7A). Similarly, T59A mutant was less potent,
while T59E mutant was more potent, than WT-nBP1a to induce the Fasn promoter in luciferase reporter assays (Fig. 7B). The increased function of T59E mutant is likely due to the increased stability/levels of this mutant. In addition, overexpression of PKM2 could further increase the transcriptional output of nBP1a-WT, but not T59A mutant (Fig. 7C). Consistent with the gene expression data, PKM2 overexpression in BP1-KO HepG2 cells expressing nBP1a-WT resulted in significantly more lipid accumulation than in those expressing nBP1a-T59A (Fig. 7D and 7E). We also established stable cell-lines that overexpress Flag-nBP1a (WT, T59A or T59E) or Flag-PKM2 (WT) in BP1-KO HepG2 cells. When compared to HepG2 cells expressing nBP1a-WT, those expressing T59A mutant were less proliferative in three-dimension (3D) culture, while those expressing T59E mutant were more proliferative (Fig. 7F), correlated with the pattern of nBP1 protein stability/levels (Fig. 3C).

Together, these results suggest a model in which PKM2 interaction with nuclear SREBP-1a increases SREBP-target gene expression and subsequent lipid biosynthesis in cancer cells, which is at least in part correlated with cancer cell proliferation.

**Blocking PKM2 binding to SREBP1a by a small peptide inhibits lipogenic gene expression**

To further determine the role of PKM2 binding to nBP1a, we synthesized eight small peptides covering the aa1-60 sequence of SREBP-1a and a control peptide (termed CP) (Fig. 8A). The C-terminal ends of those peptides were fused to a cell-penetrating sequence (GRKKRRQRRR) from the Tat proteins to enhance the efficiency of entering into cells. We first examined their effects on the interaction between PKM2 and nBP1a by Co-IP. As shown in Fig. 8A, Peptide #8 (termed P8), which corresponds to the aa43-56 sequence of SREBP-1a, could efficiently block endogenous PKM2 binding to overexpressed HA-tagged nBP1a in HepG2 cells. Consistent with the data that PKM2 interaction with nBP1a increases the nBP1a protein stability, P8 could inhibit PKM2 overexpression –induced accumulation nBP1a proteins (Fig. 8B). To determine whether those peptides affect SREBP-target gene expression, we examined gene expression in HepG2 cells after the treatment with those peptides. Consistent with the Co-IP data, only P8 significantly reduced the Fasn promoter activity in luciferase reporter assays (Fig. 8C). Similarly, only P8 significantly decreased the mRNA levels of endogenous SREBP-1a target genes, including Acc1, Acly, Fasn, Hmgcr, Ldlr, Scd1 and Gpat, as detected by qRT-PCR (Fig. 8D). Moreover, P8 efficiently inhibited wild-type, but not T59A mutant, nuclear SREBP-1a –induced activation of the Fasn promoter in luciferase reporter assays (Fig. 8E). Thus, we generated a small peptide inhibitor that is able to functionally block the interaction between PKM2 and nBP1a, likely through a mechanism of competition, and our data also suggest that the aa43-56 sequence of SREBP-1a constitutes a binding surface for PKM2 (Fig. 8F).

**Discussion**

In this study, we identified a functional link between oncogenic PKM2 and lipogenic transcription factor SREBP-1a. PKM2 increases the stability of nuclear SREBP-1a proteins by binding to SREBP-1a aa43-56. PKM2 binding to nuclear SREBP-1a also enhances the phosphorylation of SREBP-1a at the T59 residue. As a result, PKM2 activates lipogenic gene expression and lipid synthesis, and promotes SREBP1-dependent cancer cell proliferation in cell culture and in xenograft mouse models. Clinically, T59 phosphorylation of nuclear SREBP-1a in tumors is inversely correlated with the overall survival of patients with hepatocellular carcinoma, and the protein levels of PKM2 and SREBP-1 are correlated in HCC tumors, suggesting that the SREBP-1a/PKM2 complex may play a significant role in HCC. Using the biochemical information, we developed a small peptide to disrupt the SREBP-1a/PKM2 complex. Interestingly, this small peptide inhibitor could inhibit SREBP-target gene expression in cancer cells. Thus, our data reveals a novel mechanism of PKM2 regulating cancer cell proliferation by stimulating SREBP1-dependent lipid biosynthesis.

Among the transcriptional activators of lipogenic genes, SREBP-1a is highly expressed in many cancer cell-lines and multiple types of cancer, including endometrial cancer, colorectal cancer and pancreatic cancer (11,26,27). The SREBP-1a level is also correlated with cancer progression and metastasis (28,29). Moreover, the SREBP1-dependent lipogenesis is critically involved in p53 mutation -associated changes of mammary tissue architecture and tumorigenesis of breast cancer (8). Thus, the
SREBP-1a isoform may be more involved in the developmental processes and tumor growth. Consistent with the oncogenic roles of PKM2 and SREBP-1a, our data shows that PKM2 only interacts with SREBP-1a, but not SREBP-1c or SREBP-2 (Fig. 1F). The abundance of transcriptionally active SREBP-1a is controlled at the multiple steps, including transcription, proteolytic processing of the precursors and protein stability of the nuclear forms. In addition to the transcriptional up-regulation of Srebf1a in cancer cells, we have recently shown that increasing the nuclear SREBP-1a abundance by p54

\textsuperscript{arb}/NONO promotes tumorigenesis of breast cancer (30). Multiple lines of evidence in the present study indicate that PKM2 also increase the nuclear SREBP-1a protein stability. Thus, regulation of nuclear SREBP-1a protein stability may be an important mechanism in controlling cancer cell proliferation.

Previous studies have demonstrated that nuclear SREBP proteins are degraded through a ubiquitination-proteasome mechanism (22), in which phosphorylation of nuclear SREBP-1a at T426 by GSK3\(\beta\) (22) or CDK8 (23) provides the docking surface for the E3 ligase Fbw7. Although the mechanism(s) underlying PKM2 regulation of nuclear SREBP-1a protein stability are currently unknown, T59 phosphorylation is apparently correlated with the increased stability of nuclear SREBP-1a. Thus, PKM2 regulation of nuclear SREBP-1a abundance is less likely through these mechanisms. Currently, it is unclear how T59 phosphorylation controls the nuclear SREBP-1a protein stability, but phosphorylation at T59 may change SREBP-1a conformation to make it less accessible to Fbw7, as T59 phosphorylation of nuclear SREBP-1a dramatically reduces its ubiquitination. Although PKM2 can phosphorylate SREBP-1a at T59 \textit{in vitro}, future studies are necessary to further study the kinase(s) that are responsible for T59 phosphorylation. Nevertheless, the accumulated nuclear SREBP-1a proteins seem functional, as we observed an increase of lipogenic gene expression and lipid accumulation in cancer cells. Importantly, our data shows that the levels of T59-phosphorylated SREBP-1a are closely correlated with the overall outcome of HCC in human patients, suggesting the clinical significance of T59 phosphorylation of SREBP-1a.

In summary, this study uncovered a novel SREBP-1a/PKM2 protein complex, which promotes cancer cell proliferation by increasing the abundance of nuclear SREBP-1a and subsequent lipid biosynthesis. Our data suggests that the SREBP-1a/PKM2 complex may represent a novel target for fighting some types of cancer.

**Experimental procedures**

**Tissue culture and antibodies**

HEK293T, HepG2, SW480, A549 and MCF7 cell lines were purchased from ATCC and maintained in DMEM or RPMI with 25 mM glucose supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 \(\mu\)g/ml streptomycin sulfate (GIBCO). All cells were cultured in humidified incubators at 37\(^\circ\)C and 5% CO\(_2\). For gene expression experiments, cells were cultured in lipid-depleted medium. The following antibodies were used: From Sigma-Aldrich, anti-Flag M2; From Covance, anti-HA; From Santa Cruz Biotech, anti-SREBP-1; From Cell Signaling, anti-\(\beta\)-actin, anti-PKM2 and anti-phospho-serine (or threonine). MG132 and cycloheximide were purchased from Sigma-Aldrich. Lipofectamine 2000 from Invitrogen was used in transfection according to the manufacturer’s protocol.

**Co-Immunoprecipitation (Co-IP)**

For endogenous proteins, 5 \(\mu\)l of anti-SREBP1 antibodies were bound to 20 \(\mu\)l mixed Protein A/G beads (Thermo scientific) and incubated with 1 ml of cell extracts in IP buffer for 3 hr at 4\(^\circ\)C. For overexpressed Flag or HA-tagged proteins, 30 \(\mu\)l of anti-Flag M2 affinity gel (Sigma-Aldrich) or anti-HA agarose (Thermo scientific) was used. The beads were washed for five times with IP buffer. Eluted proteins were subjected to SDS-PAGE and analyzed by immunoblotting using relevant antibodies.

**GST pull-down assay**

GST-fused PKM2 or SREBP-1a full-length or mutants were expressed in \textit{E. coli} BL21 and purified using glutathione-sepharose 4B beads (GE Healthcare) according to the manufacturer’s protocol. A small amount of proteins was used to verify its expression by coomassie blue staining. 6xHis-tagged proteins were prepared and purified using Ni-affinity resins (GE Healthcare). Recombinant 6xHis-
nSREBP1 or cell lysates mixed with GST or GST-PKM2 beads at 4°C for 1 hr. The beads were then washed with IP buffer for five times. The resulting beads were analyzed by immunoblotting using relevant antibodies.

**Immunoblotting**

Cells were lysed into the RIPA buffer containing protease inhibitor cocktails and ALLN by incubating on ice for 30 min followed by centrifugation at 10,000xg for 15 min. The extracted proteins were subjected to electrophoresis on SDS-PAGE and transferred to PVDF membranes (GE Healthcare), which were blocked and probed with specific primary antibodies with appropriate dilution at 4°C overnight. After three washes with 1xTBST buffer, the membranes were then incubated with the horseradish peroxidase conjugated secondary antibodies for 1 hr at room temperature, followed by three washes with 1xTBST buffer, the immunoreactive bands were visualized using the ECL Plus System.

**Transfection of siRNA**

Cells were transfected with oligo siRNAs using Lipofectamine 2000. The sense sequences of siRNA oligos used are as follows:

- **Pkm2 #1**, AGGCAGAGGCUGCACUCUATT;
- **Pkm2 #2**, CCAUAUACGUCCUCACCAATT;
- **Pkm2 #3**, GCCCGAGGCUUCUUCAAGAAGTT;
- **Srebf1**, GCUGAAUAAAUCUGCUGUCUUTT;
- **NS control**, UUCUCCGAACGUGUCACGUTT.

**Peptide design and synthesis**

The small peptides were designed based on the aa1-60 sequence of SREBP-1a:

- **P2**, MDEPPFSEAALEQA;
- **P3**, EAALEGALGEPCDL;
- **P4**, LGEPCDLDAALLTD;
- **P5**, DAALLTDIEDMLQL;
- **P6**, IEDMLQLINNQDSD;
- **P7**, INNQDSDFPGLFDP;
- **P8**, FPGLFDPPYAGSGA;
- **P9**, PYAGSGAGGTD.

A transmembrane domain (YGRKKRRQRRR) was conjugated to C-terminus of each peptide to increase the cell-penetration. All peptides were synthesized and reversed-phase HPLC purified to >98% by China Peptides (Shanghai, China), and their molecular weights were verified by electrospray ionization mass spectrometry.

**Luciferase reporter assay**

A plasmid containing the human *Fasn* promoter (175bp) fused to the firefly luciferase gene in the pGL3 vector (Promega) was used. HepG2 cells with a density of 1x10⁴ per well were cultured in 24-well tissue culture plates and co-transfected with the firefly luciferase plasmid and a renilla luciferase plasmid (as the control) at a ratio of 10:1 in addition to over-expression plasmids or siRNA. About 24 hr post-transfection, cell lysates were analyzed by the Dual-Luciferase Assay system (Promega) according to the manufacturer’s instructions. The ratio of firefly luciferase to renilla activity was calculated for each of the triplicates.

**Real-time RT-PCR (qRT-PCR)**

Total RNA was isolated using a Trizol kit (Omega) and converted to cDNA with a cDNA synthesis kit (Takara). Quantitative real-time PCR was performed using a SYBR Green PCR Master Mix (Takara) and the transcript levels were detected by using the StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR primer sequences are available upon request.

**Proliferation and colony formation assay**

Cells were plated in 24-well plates with a density of 2x10⁴ cells per well. One day after transfection, cells were harvested every 24 hr and live cells were counted using a hemocytometer after trypan blue exclusion. For colony formation assays, cells with various treatments were suspended in culture medium containing 0.3% agarose (Sigma-Aldrich) and placed on top of solidified 0.6% agarose in 24-well plates. After culture for 14 days, colonies were stained with 0.25% crystal violet and three random regions for each treatment were analyzed under a microscope for the number of colonies.

**Three-dimension (3D) culture**

For 3D cell culture, type I collagen (Cell Matrix, Japan), F12 medium and the reconstitution buffer were mixed in ice-cold water with a ratio of 8:1:1. Cell suspension was added to the collagen solution with a final density of 1.5x10⁵ cells/ml. Three drops of the collagen-cell mixture (30 µl per drop) were dropped in each well of a 6-well plate on ice, which
was placed at 37°C in the incubator containing 5% CO2 for 1 hr to gelate the collagen-cell mixture. The final concentration was about $3 \times 10^5$ cells per collagen gel droplet. For each well, 3 ml of DF medium (Nissui Pharmaceutical) containing 10% FBS was added and the plates were incubated in a CO2 incubator at 37°C overnight. The culture medium was changed every other day. At the end of the experiment, 30 µl of Neutral Red (Kurabo, Japan) solution was added to each well. After incubation at 37°C for 2 hr, cells were washed twice with 1xPBS, fixed 20 min in water, air dried and analyzed by a high-resolution Microscope (VH-5910, Keyence, Japan).

**SREBP-1 knockout**

The CRISPR/Cas9 target sequence for Srebf1 was TCCAAAGCGGCTCGCTGAA. Self-complementary gRNA oligos corresponding to the target site were synthesized by Sangon Biotech (Shanghai, China), annealed and ligated into pCas9/gRNA using T4 DNA ligase (New England Biolabs). HepG2 cells were transfected with pCas9/gRNA-Srebf1 plasmids using Lipofectamine 2000 before selection with 1 µg/ml puromycin. Single colonies were isolated by limited dilution and expansion. Clones were then genotyped by sequencing and validated by immunoblotting.

**Lipid staining**

Two days after transfection, HepG2 cells were washed for three times with 1xPBS, and then fixed with 4% formalin for 20 min at room temperature. After gently rinse with 1xPBS for 3 times, cells were incubated with 1x LipidTOX (Invitrogen) for 30 min at room temperature. Images were examined and recorded by an Axio Observer Z1 microscope.

**Xenograft tumor studies**

All experimental procedures using animals were in accordance with the protocol approved by the Animal Ethics Committee of Renji Hospital of Shanghai Jiao Tong University School of Medicine. Tumor cells ($5 \times 10^6$ per mouse) were harvested by trypsinization, resuspended in culture medium and Matrigel (BD Bioscience), and inoculated subcutaneously in the flank of 4-week-old male BALB/c SCID mice (Shanghai Laboratory Animal Center, Shanghai, China). Eight to ten mice were used in each group. Tumor growth was assessed twice a week with caliper measurement. Tumor volume was calculated according to the following formula: $V = (\text{Length} \times \text{Width}^2)/2$. At the end of the experiments, mice were euthanized and tumor tissues were harvested and weighed. For peptide treatments, mice were randomly divided into two groups when tumor mass was established (approximately 50 mm in diameter).

**Immunohistochemistry**

All experiments involving human tissues were approved by the Human Assurance Committee of Renji Hospital of Shanghai Jiao Tong University School of Medicine, and were performed in compliance with the Declaration of Helsinki. All tissue samples were obtained from surgical resections agreed by patients of Renji Hospital. For immunohistochemical analyses, sections were dewaxed, hydrated, and washed. After microwave antigen retrieval, the slides were treated with 3% H2O2 to block endogenous peroxidase activity and then incubated overnight with anti-PKM2 antibody (1:3000), anti-p-T59 of SREBP-1 (1:1000) or anti-SREBP-1 antibody (1:500). The sections were then incubated with the horseradish peroxidase-conjugated secondary antibody and the signals were visualized with diaminobenzidine as the chromogen and counter-stained by hematoxylin. Two independent researchers blandly scored the signal intensity of immunohistochemistry without prior knowledge about the patient and sample. The signal intensity was scored on a scale of 0–3: 0 = negative, 1 = weak, 2 = moderate and 3 = strong. The percentage of stained cells was also scored on a scale of 0–3: 0 = no staining, 1 = <25%, 2 = 25–50% and 3 = >50% staining. Thus, the final scores for indicated protein in those liver tissues were on a scale of 0–9, in which a score of 3 or more was defined as high expression and a score less than 3 as low expression.

**Statistical analysis**

All data were statistically analyzed using Graphpad Prism 6 software or SPSS 20, and presented as Mean ± SEM. Unpaired two-tailed t-test or ANOVA test was used to analyze the difference between two groups. Multivariate logistic regression was used to identify independent risk factors for the expression of p-T59 phosphorylation of SREBP-1 proteins in human liver tissues. Spearman's rank correlation was applied to determine the correlation.
between PKM2 and SREBP-1 proteins. P-values < 0.05 were considered as statistically significant.

Author contributions–X.Z., F.Y. and G.H. conceived and designed the experiments; X.Z., J.Liu, L.Z., H.Y., J.Li and X.M. performed the experiments; X.Z., F.Y., J.Liu and G.H. wrote the manuscript.

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Table 1. SREBP-1 phosphorylation at T59 in HCC.

| Variable               | T59 Phosphorylation | N | Low | High | p Value |
|------------------------|---------------------|---|-----|------|---------|
| **Age (years)**        |                     |   |     |      |         |
| < 50                   |                     | 31| 16  | 15   |         |
| ≥ 50                   |                     | 59| 20  | 39   | 0.896   |
| **Gender**             |                     |   |     |      |         |
| Female                 |                     | 9 | 4   | 5    |         |
| Male                   |                     | 81| 32  | 49   | 0.142   |
| **Tumor size (cm)**    |                     |   |     |      |         |
| ≤ 5                    |                     | 36| 15  | 21   |         |
| > 5                    |                     | 54| 21  | 33   | 0.085   |
| **Histologic grade**   |                     |   |     |      |         |
| I, II                  |                     | 64| 32  | 32   |         |
| III                    |                     | 26| 4   | 22   | 0.008   |
| **TNM stage**          |                     |   |     |      |         |
| I-II                   |                     | 38| 21  | 17   |         |
| III-IV                 |                     | 52| 15  | 37   | 0.030   |
| **Liver Cirrhosis**    |                     |   |     |      |         |
| +                      |                     | 57| 23  | 34   |         |
| -                      |                     | 33| 13  | 20   | 0.629   |
Figure 1. SREBP-1a interacts with PKM2.

A. Silver staining of nBP1a–binding proteins that were immunoprecipitated from nuclear extracts of HEK293T cells stably transfected with Flag-nBP1a.
B. Co-IP analysis of endogenous PKM2 binding to overexpressed Flag-nBP1a in HepG2 cells. The presence of PKM2 in IP elutes was analyzed by immunoblotting using anti-PKM2 antibody.
C. Co-IP analysis of overexpressed HA-PKM2 binding to overexpressed Flag-nBP1a in HepG2 cells.
D. Immunostaining to analyze the localization of co-transfected HA-PKM2 and Flag-nBP1a in HepG2 cells.
E. A diagram shows the sequence difference between SREBP-1a and SREBP-1c.
F. Co-IP analysis of endogenous PKM2 binding to overexpressed Flag-tagged nuclear forms of SREBP-1a, SREBP-1c and SREBP-2 in HEK293T cells.
G. Co-IP analysis of overexpressed HA-PKM2 binding to endogenous nuclear form of SREBP-1 in HepG2 cells.
H. Co-IP analysis of endogenous PKM2 binding to endogenous nuclear form of SREBP-1 in HepG2 cells.
I. GST pull-down analysis of interaction between recombinant PKM2 and SREBP-1a.

Figure 2. Nuclear SREBP1 protein stability is enhanced by PKM2.

A. Sections of human HCC tissues were stained with anti-SREBP1 or anti-PKM2 antibody. Representative tissue images are shown (Scale bar, 50 µm).
B. Semi-quantitative analyses of immunohistochemistry data of human HCC tissues for PKM2 or SREBP1. Correlation between PKM2 and SREBP1a was analyzed by Spearman rank correlation analysis.
C. Effects of PKM2 knockdown by siRNA on endogenous SREBP-1 protein levels in HepG2 cells.
D. Effects of PKM2 knockdown on the degradation of overexpressed Flag-nBP1a in the presence of cycloheximide (CHX).
E. Semi-quantitative analyses by densitometry of the effects of PKM2 knockdown on the degradation of overexpressed Flag-nBP1a (n=3).
F. Effects of the proteasome inhibitor MG132 on PKM2 regulation of overexpressed Flag-nBP1a.

Figure 3. T59 phosphorylation of nuclear SREBP-1a enhances its protein stability and binding to PKM2.

A. Effects of PKM2 knockdown on the phosphorylation of nuclear SREBP-1 in HepG2 cells.
B. GST pull-down analyses of Flag-PKM2 binding to the indicated fragments of SREBP-1a.
C. Immunoblotting of the indicated mutants of Flag-nBP1a in HepG2 cells.
D. Effects of PKM2 knockdown on the protein levels of wild-type and T59A mutant Flag-nBP1a.
E. Effects of PKM2 knockdown on the phosphorylation of Flag-nBP1a at threonine residues. (The immunoprecipitated Flag-nBP1a proteins were normalized to the same level before comparison for phosphorylation.)
F. Effects of PKM2 knockdown and T27A or T59A mutation on Flag-nBP1a ubiquitination.
G. Effects of PKM2 knockdown on the degradation of overexpressed wild-type and T59A mutant Flag-nBP1a in the presence of cycloheximide (CHX) (n=3).
H. Effects of PKM2 on SREBP-1a phosphorylation by in vitro kinase assays.
I. Effects of wild-type and K367M mutant PKM2 on wild-type, T27A and T59A mutant SREBP-1a phosphorylation in vitro.
Figure 4. Phosphorylation of SREBP-1a at the T59 residue predicts the poor prognosis of HCC.

A. Validation of the anti-phosph-T59-SREBP-1 antibody by immunohistochemistry analyses of HepG2 cells with transfections of vector, wild-type (WT) or T59A mutant of nBP1a.
B. Effects of pretreatment with non-phophopeptides and phosphopeptides on the immunohistochemistry signals, which were generated using the anti-phosph-T59-SREBP-1 antibody, in liver samples from HCC patients.
C. Representative images of histopathologic sections of human HCC and adjacent normal tissues that were analyzed by immunohistochemistry using anti-phosph-T59-SREBP-1 antibody or H&E staining. Low and high staining groups were categorized according to the immunohistochemistry scores of SREBP-1 T59 phosphorylation. (Scale bar, 50 µm).
D. Kaplan–Meier analysis of overall survival in correlation with the levels of SREBP-1 T59 phosphorylation.
E. Semi-quantitative analyses of immunohistochemistry data of human HCC and adjacent normal tissues for SREBP-1 T59 phosphorylation.
F. Correlation analysis of staining score of SREBP-1 T59 phosphorylation and tumor grade of human HCC.
G. Correlation analysis of staining score of SREBP-1 T59 phosphorylation and tumor stage of human HCC.

Figure 5. PKM2 regulates SREBP1-dependent cancer cell growth.

A. Effects of SREBP1 depletion and PKM2 overexpression on cell growth of HepG2, SW480, A549 or MCF7 cells.
B. Effects of the indicated compounds on PKM2-induced increase of HepG2 cell growth.
C. Generation of SREBP1-knockout HepG2 cells.
D. Effects of overexpressed wild-type or the indicated mutant nBP1a on HepG2 cell growth.
E. Growth curve of xenograft tumors from SREBP-1 knockout HepG2 cells that were stably transfected with vector control, PKM2, wild-type or T59A mutant nBP1a in nude mice.
F. Weight of tumors from stably transfected SREBP-1 knockout HepG2 cells when mice were sacrificed.
G. Effects of PKM2 knockdown on HepG2 cell growth. (* p<0.05, ** p<0.01 vs. control; n.s.: not significant)

Figure 6. PKM2 stimulates lipid biosynthesis in cancer cells.

A. Real-time RT-PCR analysis of the effects of PKM2 knockdown in HepG2 cells by three independent siRNA (NS-siRNA as control) on the mRNA of indicated genes. (All significantly different except the indicated)
B. Effects of PKM2 knockdown in HepG2 cells by siRNA on the activity of human Fasn promoter as detected by luciferase reporter assay.
C. Effects of PKM2 depletion by siRNA on cellular lipid levels in HepG2 cells, which were stained with LipidTOX (red). The nuclei were stained with Hoechst3342 (blue). (Scale bar, 50 µm).
D. Effects of PKM2 depletion on cellular triglyceride levels in HepG2 cells.
E. Effects of PKM2 depletion on cellular cholesterol levels in HepG2 cells.
F. LC-MS analysis of the effects of PKM2 depletion on fatty acid profiles in HepG2 cells. (All significantly different except the indicated)
G. Real-time RT-PCR analysis of the effects of SREBP1 depletion and PKM2 overexpression in HepG2, SW480, A549 or MCF7 cells on the mRNA of indicated genes.

H. Effects of SREBP1 depletion and PKM2 overexpression in HepG2, SW480, A549 or MCF7 cells on the activity of human *Fasn* promoter as detected by luciferase reporter assay. (* p<0.05, ** p<0.01, *** p<0.01 vs. control; n.s.: not significant)

**Figure 7.** T59 phosphorylation of nuclear SREBP-1a promotes lipid biosynthesis and cancer cell proliferation.

A. Real-time RT-PCR analysis of the effects of overexpressing wild-type (WT), T59A or T59E mutant nBP1a in SREBP1-knockout cells on the mRNA levels of indicated genes.

B. Effects of overexpressing wild-type (WT), T59A or T59E mutant nBP1a in SREBP1-knockout cells on the activity of human *Fasn* promoter as detected by luciferase reporter assay.

C. Effects of PKM2 overexpression on wild-type or T59A mutant Flag-nBP1a -induced activation of human *Fasn* promoter in HepG2 cells as examined by luciferase reporter assays.

D. Effects of PKM2 overexpression and/or T59A mutation in nBP1a on lipid accumulation HepG2 cells. Cellular lipids were stained with LipidTOX, and nuclei were stained with Hoechst3342. (Scale bar, 50 µm)

E. Effects of PKM2 overexpression and/or T59A mutation in nBP1a on triglyceride levels in HepG2 cells.

F. Effects of overexpressed wild-type, T59A or T59E mutant nBP1a in SREBP-1 knockout HepG2 cell growth in 3D culture. (*** p<0.001, n.s. p>0.05 vs. vector alone; # p<0.01, & p<0.001 vs. T59A-nBP1a alone, n=3)

**Figure 8.** Blocking nBP1a binding to PKM2 by small peptides inhibits gene expression.

A. Co-IP analysis of overexpressed Flag-PKM2 binding to HA-nBP1a in HepG2 cells in the presence of peptides P2-P8 (8 µM) or without peptide (CT: peptide solvent control; None: no-treatment control). The lower panel shows the peptide sequences corresponding to aa1-60 of SREBP-1a.

B. Effects of 8 µM peptide P8 on overexpressed HA-nBP1a protein levels in HepG2 cells.

C. Effects of peptides P2-P9 (8 µM) on the *Fasn* promoter activity in HepG2 cells as detected by luciferase reporter assay.

D. Effects of treatment with peptides P2-P9 (8 µM) for 24 hr on the mRNA levels of indicated genes in HepG2 cells.

E. Effects of P8 or control peptide CP (8 µM) on the *Fasn* promoter activity upon overexpressing wild-type or T59A mutant SREBP-1a in HepG2 cells as detected by luciferase reporter assay.

F. A suggested model shows how the small peptide P8 may work.
Figure 2

A

Case A

Case B

Case C

B

PKM2 Level

SREBP1 Level

p = 0.2672; p < 0.05

C

siRNA: NS  Pkm2

Pre-BP1  135

nBP1  100

PKM2  75

Actin  35

D

siRNA: NS  Pkm2

CHX (hr): 0 0.5 1.0 2.0 0 0.5 1.0 2.0

Flag (nBP1a)  75

Actin  63

E

Relative Protein levels

Hours after Adding CHX

F

siRNA: NS  Pkm2

MG132: - - + +

Flag (nBP1a)  75

Actin  63

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Figure 3
Figure 4

A

Anti-p-T59-BP1

Vector

Flag-nBP1α-WT

Flag-nBP1α-T59A

B

Anti-p-T59-BP1

No Peptide

+ Phosphopeptide

+ Non-phosphopeptide

C

HCC

Normal

p-T59-BP1

H&E

p-T59-BP1

H&E

Case 1

Case 2

Case 3

D

Survival Rate (%)

0 20 40 60 80

Months

0

2

4

6

8

Low score

High score

E

Staining Score

p<0.01

p<0.01

p<0.05

F

G

Normal

HCC

Grade I, II

Grade III

Stage I, II

Stage III, IV

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Figure 6

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

http://www.jbc.org/Downloaded from
Figure 7

A. Relative RNA Levels

B. Fasn-Luc Relative Activity

C. Fasn-Luc Relative Activity

D. Hoechst and Lipids

E. Triglycerides

F. WT, T59A, T59E (Flag-nBP1α Overexpression)
Figure 8

A

Flag (PKM2)
HA (nBP1a)

P2
P3
P4
P5
P6
P7
P8
P9

AA1-60
(IP: HA)/WB

D

Relative mRNA Levels

Acc1  Acy1  Fasn  Hmgcr  Ldlr  Scd1  Gpat

CT  P2  P3  P4  P5  P6  P7  P8  P9

B

Flag-PKM2:
HA (nBP1a)
Actin

C

Fasn-Luc

Relative Activity

CT  P2  P3  P4  P5  P6  P7  P8  P9

E

Fasn-Luc

Relative Activity

nBP1-WT  nBP1-T59A

F

PKM2

SREBP-1

(- P8)  (+ P8)
Pyruvate kinase M2 interacts with nuclear sterol regulatory element-binding protein 1a and thereby activates lipogenesis and cell proliferation in hepatocellular carcinoma
Xiaoping Zhao, Li Zhao, Hao Yang, Jiajin Li, Xuejie Min, Fajun Yang, Jianjun Liu and Gang Huang

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