Hepatic IRF6 alleviates liver steatosis and metabolic disorder
by transcriptionally suppressing PPARγ

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**Key Words:**

Interferon Regulatory Factor 6, Peroxisome proliferator-activated receptor γ, non-alcoholic fatty liver disease, digital gene expression, Chromatin Immunoprecipitation

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**List of Abbreviations**

NAFLD, Nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; IRF, Interferon Regulatory Factor; HKO, hepatocyte-specific knockout; HTG, hepatocyte-specific transgenic; NTG, non-transgenic; PPARγ, Peroxisome proliferator-activated receptor γ; PPARα, Peroxisome proliferator-activated receptor α; GEO, gene expression omnibus; HFD, high fat diet; IFN, interferon; PGM3, Phosphoacetylglucosamine mutase; PNLD1, Poly(A)-specific ribonuclease PNLD1; STAP1, Signal-transducing adaptor protein 1; NC,
normal chow; HFHC, high fat and high cholesterol; PA, palmitic acid; OA, oleic acid; qPCR, quantitative real-time-PCR; ORO, oil red o; NPC, non parenchymal cells; BW, body weight; FBG, fasting blood glucose; FINS, fasting insulin; HOMA-IR, Homeostasis model assessment-insulin resistance; GTT, glucose tolerance test; AUC, area under curve; LW, liver weight; TG, triglyceride; TC, serum total cholesterol; NEFA, nonesterified fatty acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; HTG, hepatocyte-specific transgenic; WT, wild type; PCA, principal component analysis; DEG, differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes; DGE, digital gene expression; SCD1, Stearoyl-CoA Desaturase 1; CD36, fatty acid translocase; FASN, Fatty Acid Synthase; PO, palmitic acid and oleic acid; siRNA, small interfere RNA; RNA-Seq, RNA sequencing; ChIP, Chromatin Immunoprecipitation; ChIP-Seq, Chromatin Immunoprecipitation sequencing.

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Abstract:

Nonalcoholic fatty liver disease (NAFLD) has become a worldwide epidemic. A large and growing unmet therapeutic need has inspired numerous studies in the field. Integrating the published genomic data available in the Gene Expression Omnibus with NAFLD samples from rodents, we discovered that interferon regulatory factor 6 (IRF6) is significantly suppressed in high-fat diet (HFD)-induced fatty liver. In the current study, we identified IRF6 in hepatocytes as a protective factor in liver steatosis. During HFD challenge, hepatic Irf6 was suppressed by promoter hypermethylation. The severity of HFD-induced liver steatosis was exacerbated in hepatocyte-specific Irf6 knockout mice, whereas hepatocyte-specific transgenic mice overexpressing Irf6 (IRF6-HTG) exhibited alleviated responses to HFD feeding. Mechanistic studies in vitro demonstrated that hepatic IRF6 directly binds to the promoter of the peroxisome proliferator-activated receptor γ (PPARγ) gene and subsequently halts the transcription of Ppary and its target genes (e.g., genes that regulate lipogenesis and lipid acid uptake) under physiological conditions. After metabolic stimulus exposure, the insufficient production of IRF6 in hepatocytes led to a failure to inhibit Ppary and its targets, driving abnormalities of lipid metabolism.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) has become the fastest growing liver disease worldwide. The current global prevalence of NAFLD is estimated to be approximately 25%, and NAFLD is predicted to become the leading cause of end-stage liver disease and liver transplantation (1, 2). Due to the tremendous burden of managing NAFLD and its related comorbidities, the rapid discovery of effective treatments for these diseases is needed. Multiple molecular targets have been proposed and proven to be involved in the pathogenesis of NAFLD. Unfortunately, there are still no effective pharmacological therapies for NAFLD available at this time. Therefore, we analyzed the published genomic data from a murine model of high-fat diet (HFD)-induced fatty liver in the Gene Expression Omnibus (GEO) database. Interferon regulatory factor 6 (IRF6) was shown to be downregulated in HFD-induced fatty liver. Consistent with this finding, the expression of the IRF6 protein was attenuated in fatty liver tissue from both rodents and humans. However, the role of IRF6 in the pathogenesis of NAFLD has not been investigated.

IRF6 belongs to a family of transcription factors that typically possess a novel helix-turn-helix DNA-binding motif, which was first identified in the posterior mesoderm during the early development of Xenopus laevis in 1997(3). IRF6 was reported to function in the regulation of orofacial development by Murray JC in 2002(4). Later studies found that IRF6 is also necessary for the development and differentiation of epithelial-derived tissues, salivary glands and the pancreas (5-7). Global IRF6-deficient mice have severe skin abnormalities, as well as abnormal limb and craniofacial development(8). Unlike other IRFs, IRF6 has rarely been reported to be involved in the regulation of interferons (IFNs) in higher-level vertebrates (9). Considering the important role of IRF6 in embryonic development(8) and its dramatic suppression in fatty liver disease, we speculate that eliciting IRF6 signaling might have an important effect on NAFLD.

In this study, we tested the hypothesis that attenuated HFD-induced hepatic steatosis is mediated by a hepatic IRF6-dependent mechanism. We found that the expression of hepatic Irf6 was suppressed by promoter hypermethylation in mice fed a HFD. In vivo studies
confirmed that the deletion of *Irf6* in hepatocytes exacerbates hepatic steatosis; conversely, the overexpression of hepatic *Irf6* mitigates metabolic abnormalities in the liver. Mechanistically, IRF6 binds primarily to the steatogenic gene *Pparγ* and inhibits its transcription. This regulation largely inhibits lipogenesis and lipid uptake/storage. These data provide evidence for a major role of hepatic IRF6 and its transcriptional signature in HFD-induced liver steatosis.

Methods and Materials

**Generation of Hepatocyte-Specific IRF6 Knockout/Transgenic Mice**

IRF6\textsuperscript{Flox/Flox} (IRF6-Flox) mice were created using the CRISPR/Cas9 system, and are detailed in the Supporting Information.

**Establishment of NAFLD Model in Mice**

Mice husbandry and metabolic assessment are detailed in the Supporting Information.

**Human Liver Sample**

Human liver samples including normal and NAFLD were collected from of Zhongnan Hospital Liver Disease Resource Library of Wuhan University. The Ethics Committee of Zhongnan Hospital of Wuhan University approved the procedure involving human samples according to the guidelines of the Declaration of Helsinki.
**Metabolic Parameter Analysis**

Fasting serum insulin levels, fasting blood glucose levels, homeostatic model assessment of insulin resistance (HOMA-IR) values, insulin tolerance tests (ITTs) and glucose tolerance tests (GTTs) were examined and performed as described (10).

**Liver Lipid and Function Measurements**

Liver lipid were measured using commercial kits and detailed in the Supporting Information.

**Histological Analysis and Immunofluorescence Staining**

Histological experiments were described and detailed in the Supporting Information.

**Primary hepatocytes Isolation and Culture**

Primary hepatocytes were isolated from 6- to 8-week-old male C57BL/6J or IRF6-HepKO mice using a two-step collagenase perfusion method, and detailed in the Supporting Information.
**Nucleus and Cytosol Separation**

Primary hepatocytes was collected and separated with detail description in the Supporting Information.

**Small-interfering RNA Transfection**

L02 cells were transfected with small-interfering RNA with detail description in the Supporting Information.

**Western Blot Analysis**

Western Blotting was used to detect the expression of protein, and detailed in the Supporting Information.

**Quantitative Real Time-PCR (qPCR)**

Total RNA was extracted with Trizol and following the detail procedure in the Supporting Information.
**DNA Methylation Sequencing**

Genomic DNA was extracted from livers using the DNeasy Blood and Tissue Kit, following the detail procedure in the Supporting Information.

**RNA Sequencing**

Single-end RNA libraries prepared from liver of Flox and IRF6-HKO mice with a high fat diet for 10 weeks were sequenced using BGISEQ-500 in Beijing Genomics Institute, and detailed in the Supporting Information.

**Chromatin Immunoprecipitation (ChIP) and ChIP-Sequencing**

L02-3xFlag-IRF6 cells were cross-linked and collected for ChIP, following the detail procedure in the Supporting Information.

**Statistical Analysis**

Statistical analysis was performed as described (22), and detailed in the Supporting Information.
Results

IRF6 expression is downregulated in the fatty liver

To determine the gene expression profile associated with NAFLD, we performed a comprehensive analysis of the publicly available genomic data generated from liver tissue from C57BL/6J mice fed with normal chow diet or high fat diet in the GEO database (Supporting Table S1). Both upregulated and downregulated genes were identified by differentially expressed gene (DEG) analysis in each comparative group (Fig. 1A). Four genes with consistent pattern changes across six databases were discovered by intersection analysis: *Irf6*, *Pgm3*, *Pnldc1* and *Stap1* (Fig. 1B). Interestingly, *Irf6* was the only gene downregulated in the HFD groups, which indicates that IRF6 is probably suppressed via a specific mechanism in mice fed a HFD (Fig. 1C). Consistent with the results obtained by mining the GEO database, mRNA expression of *Irf6* was attenuated in the liver in both HFD-fed (Fig. 1D) and high-fat high-cholesterol (HFHC)-fed mice (Fig. 1E). Western blot analysis also showed that the expression of IRF6 began to decrease from the early stage (Supporting Fig. S1A-D) and decreased significantly after 24 weeks of HFD feeding (Fig. 1F) or 16 weeks of HFHC feeding in mice (Fig. 1G), accompanied by the gradual development of fatty liver. This demonstrated that decreased expression of IRF6 could be a cause of enhanced fatty liver at the early stage rather than a result of HFD feeding. Cells expressing IRF6, as detected by immunohistochemistry, were decreased in liver sections from HFD-fed mice (Fig. 1H) and patients with NAFLD (Fig. 1I, Supporting Table. S2). We also examined the expression of other IRF family members during HFD feeding, except for *Irf1* and *Irf8*, six
other family members have significantly decreased mRNA expression after HFD (Supporting Fig. S1E). This result may imply the important role of IRFs in NAFLD. Taken together, the above evidence showed a significant decrease in IRF6 in the context of NAFLD, indicating its potential role in the pathogenesis of this disease.

**Hepatic expression of *Irf6* is suppressed by promoter hypermethylation in mice fed a HFD**

We further explored the potential mechanism that attenuates *Irf6* expression in the fatty liver. Primary hepatocytes cells were treated with palmitic acid (PA) and oleic acid (OA), well-established inducers of lipid accumulation (11, 12), for 24 h to further confirm that excessive lipid challenge decreases *Irf6* expression *in vitro*. The mRNA (Fig. 2A) and protein (Fig. 2B) expression of IRF6 was attenuated by PA and OA (PO) treatment. To exclude potential protein degradation mediated by proteases, MG132, a potent proteasome inhibitor, was applied simultaneously with PO, and the decreases in IRF6 protein expression were not rescued (Fig. 2C). Interestingly, the expression of *Irf6* increased by exposed to inflammatory and oxidative stress factors in primary hepatocytes which indicated that hepatic lipid accumulation in NAFLD is the key inducer of *Irf6* decreased expression (Supporting Fig. S2A, S2B).

Methylation of CpG islands in the promoter region is considered a common means of silencing tumor suppressor genes(13). Previous studies showed that a large number of genes are hypomethylated or hypermethylated in the liver during HFD challenge (14). To determine whether the decrease in *Irf6* expression is mediated by promoter

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hypermethylation, we screened the Irf6 gene for potential CpG islands using the MethPrimer program (15). We found two CpG islands, which contain 28 CpG sites, located within the regions from -278 bp to -36 bp and 60 bp to 185 bp (Fig. 2D). The presence of these CpG islands was also confirmed by the University of California Santa Cruz (UCSC) Genome Browser (16). To further examine the methylation status within the CpG islands, the presence of 5-methyl cytosine, as a methylation marker, was detected. Methylation of 7.86% of the CpG sites in the CpG islands was detected in the HFD group compared to methylation of 1.07% of the CpG sites in the normal diet control (NC) group (Fig. 2E). Moreover, the expression of DNA methylase increased significantly in HFD group (Supporting Fig. S2C). To determine if hepatic Irf6 is suppressed by promoter hypermethylation during lipid challenge, primary hepatocytes were co-treated with PO and 5-azacytidine (5-AzaC), a DNA methyl transferase inhibitor. Because of the basal DNA methylation, 5-AzaC prevented PO-induced decreases even a slight increases in Irf6 expression at the mRNA and protein levels compared to BSA group (Fig. 2F, G). Thus, the methylation status of the promoter region in hepatocytes determines Irf6 expression during metabolic challenge.

IRF6 prevents lipid accumulation in hepatocyte in vitro

IRF6, as a member of the IRF family, to determine whether IRF6 also functions as a transcription factor in hepatocyte, we first examined the intercellular distribution of IRF6 in liver and primary hepatocytes. Immunofluorescence staining showed that IRF6 resides in both the cytosol and nucleus but has higher intensity in the nucleus in both NC and HFD fed
mice liver tissue (Fig. 3A, Supporting Fig. S3A). A nucleus and cytosol separation assay showed the same expression pattern for IRF6 in mice primary hepatocytes (Fig. 3B). Notably, IRF6 is not completely co-localized with HNF4, marker of hepatocytes, suggesting that IRF6 localized extra-hepatocytes of the liver (Supporting Fig. S3B). Taken together, IRF6 expressed mainly in the nucleus of hepatocytes, but also in non-parenchymal cells (Kupffer cell and HSC).

To determine whether IRF6 expression in hepatocytes plays an essential role in steatosis in vitro, we established IRF6 overexpression and knockout cell lines in L02 cells (Supporting Fig. S3C and Table. S3). Oil red O staining showed that exposure to PO for 24 h induced significant lipid accumulation in the hepatocytes. IRF6 overexpression in L02 cells significantly attenuated PO-induced lipid accumulation compared to that of the controls (Fig. 3C, D). In contrast, IRF6 knockout in L02 cells augmented PO-induced lipid accumulation by 2-fold compared to that of the controls (Fig. 3E, F). The expression of genes regulating hepatic cholesterol synthesis, lipogenesis, fatty acid uptake and fatty acid oxidation were measured by quantitative real-time PCR (q-PCR) in both the IRF6 knockout cell line and the control cell line after exposure to PO. Inhibition of IRF6 significantly increased PO-induced gene expression related to cholesterol synthesis, lipogenesis and fatty acid uptake, and a minor increase was also observed in the gene regulating fatty acid oxidation (Fig. 3G). Therefore, these data suggest that IRF6 expression controls lipid metabolism in hepatocytes in vitro.

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Hepatocyte-specific deletion of IRF6 (IRF6-HKO) aggravates HFD-induced hepatic steatosis

To further determine if IRF6 in hepatocytes is critical for HFD-driven liver steatosis in vivo, we selectively depleted the Irf6 gene from the hepatocytes using the strategy described in the Supporting Materials (Supporting Fig. S4A, S4B). Representative Western blots showed that IRF6 expression was sufficiently blocked in the liver tissues subjected to hepatocyte-specific deletion of IRF6 (IRF6-HKO) compared to that in IRF6-Flox mice (Fig. 4A). The baseline metabolic profiles were not altered in IRF6-HKO mice and IRF6-HKO mice did not show a further increase in HFD-induced obesity (Fig. 4B). Compared to IRF6-Flox mice, IRF6-HKO mice exhibited a significant impairment in HFD-induced glucose resistance, as indicated by fasting blood glucose (Fig. 4C), glucose tolerance test (GTT) (Fig. 4F), and insulin resistance (HOMA-IR) (Fig. 4E). The fasting insulin level (Fig. 4D) and the insulin tolerance test (ITT) (data not shown) were not further impaired in IRF6-HKO mice fed a HFD compared to those of their IRF6-Flox counterparts. Importantly, IRF6-HKO mice exhibited an exacerbation of HFD-induced hepatic steatosis compared to IRF6-Flox mice, as indicated by liver weight (LW) (Fig. 4G), the liver/body weight (LW/BW) ratio (Fig. 4H), lipid content (triglycerides, TGs; total cholesterol, TC; and nonesterified fatty acids, NEFA) (Fig. 4J), and lipid accumulation (Fig. 4I). No further increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels was observed in IRF6-HKO mice fed a HFD compared to IRF6-Flox mice (Fig. 4K). Taken together, these data suggest that the deletion of IRF6 in hepatocytes exacerbates hepatic steatosis in vivo, indicating that hepatic IRF6 is protective against for fatty liver.

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Hepatocyte-specific overexpression of IRF6 (IRF6-HTG) mitigates HFD-induced hepatic steatosis

We further investigated whether overexpressing IRF6 in hepatocytes alleviates HFD-induced hepatic steatosis \textit{in vivo} by generating hepatocyte-specific IRF6 transgenic mice (IRF6-HTG) according to the strategy described in the Supporting Materials (Supporting Fig. S4C). Representative Western blots showed that IRF6 expression was augmented in the liver tissues from IRF6-HTG mice compared to that in the liver tissues from non-transgenic (NTG) mice (Fig. 5A). The IRF6-HTG mice also did not show a significant change in HFD-induced obesity (Fig. 5B) contrary to IRF6-NTG.

IRF6-HTG mice exhibited a significant amelioration of HFD-induced glucose resistance, as indicated by FBG (Fig. 5C), GTT (Fig. 5F) and HOMA-IR (Fig. 5E) compared with IRF6-NTG mice, accompanied by no significant difference in insulin resistance (Fig. 5D). An alleviation of HFD-induced hepatic steatosis also exhibited in IRF6-HTG mice, as indicated by LW (Fig. 5G), the LW/BW ratio (Fig. 5H), lipid content (TG, TC, and NEFA) (Fig. 5J), and lipid accumulation (Fig. 5I), with no obvious decrease in serum ALT and AST levels (Fig. 5K). These data suggest that overexpression of IRF6 in hepatocytes can alleviate hepatic steatosis \textit{in vivo}. Taken together, our loss- and gain-of-function data clearly demonstrate that IRF6 repressed hepatic lipid accumulation and HFD-induced fatty liver.
Transcriptional profile governed by IRF6 during hepatic steatosis

To further explore the downstream mechanism by which IRF6 affects hepatic steatosis, we integrated digital gene expression (DGE) analysis of hepatic RNA from HFD-fed IRF6-Flox and IRF6-HKO mice with high-resolution global binding profiles of IRF6 chromatin immunoprecipitation (ChIP) samples obtained from hepatocytes. Candidate targets were explored by intersection analysis following RNA-Seq and ChIP-Seq. The experimental strategy is shown in Fig. 6A. Based on unsupervised hierarchical clustering analysis, the gene expression profiles in IRF6-Flox and IRF6-HKO mice were clearly separated with 10w HFD fed (Fig. 6B). The criteria for the selection of genes with different expression patterns were i) Log2 (fold change) of gene expression >0.585 or <-0.585 and ii) adjusted P value <0.05. As depicted in Fig. 6c, 917 downregulated and 1090 upregulated genes were obtained (Fig. 6C). According to Gene Ontology (GO) analysis, four of the top five enriched biological processes related to IRF6 function were lipid metabolism-related processes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed for the four lipid metabolism-related processes. KEGG analysis revealed that the PPAR signaling pathway was highly enriched (Fig. 6D). This analysis scheme provides key information for determining the downstream targets of IRF6 during metabolic challenge.

To further determine which gene targets were directly bound to IRF6 in hepatocytes specially, ChIP-Seq analysis was performed in L02 cell line transfected with Flag-tagged IRF6 plasmid. Peak calling analysis performed by model-based ChIP-Seq analysis(17) yielded a highly significant value for 589 peaks, and analyses of peak positions relative to gene locations revealed the distribution of the binding sites of IRF6 (Fig. 6E). Then, a bioinformatics intersection analysis between DEGs and the directly binding genes revealed to be within TSS±3000 bp by ChIP-Seq was performed, and five genes E(PPARγ, C3, ANKRD9, SEMA4B and BRD9) were identified (Fig. 6F). PPARγ presented the highest score among the identified genes. Interestingly, PPARγ, a member of the nuclear receptor
superfamily, is a key transcription factor that regulates adipogenesis (18-21) and hepatic steatosis (22-26). As shown in Fig. 6d, the PPAR signaling pathway was dramatically changed in the IRF6-HKO group compared to that in the IRF6-Flox group. Therefore, through the integration of RNA-Seq and ChIP-Seq, we discovered that PPARγ could be the direct target of IRF6 in HFD-induced fatty liver.

**IRF6 represses PPARγ expression at the transcript level**

The high-resolution peak identified from the reading density of the ChIP-Seq data revealed a binding region spanning 3280 bp of the PPARγ gene, with a peak appearing 2244 bp downstream of the transcription start site (Fig. 7A, Supporting Fig. S5A, S5B). An independent ChIP assay was performed in L02 cells and confirmed that IRF6 binds to the PPARγ promoter directly. In contrast, gene segments outside the peak area showed negative results compared with the segments inside the peak area (Fig. 7B). The primers used are summarized in Supporting Table S3. As PPARγ is a key transcription factor of lipid metabolic genes, we investigated whether IRF6 regulated PPARγ as well as its target genes. Biological function and mRNA expression analysis of the PPAR signaling pathway showed that PPARγ target genes were significantly upregulated, especially lipogenesis-related genes (Fig. 7C). Further independent qPCR assays confirmed that PPARγ and its downstream genes, including genes involved in lipogenesis and fatty acid uptake, were upregulated/downregulated in the liver of IRF6-HKO/IRF6-HTG mice (Fig. 7D, Supporting Fig. S6).
To further establish the regulatory relationship between \textit{Irf6} and \textit{Ppar\gamma} expression, an \textit{in vitro} fatty liver model was used in combination with PO treatment. Primary hepatocytes from IRF6-Flox and IRF6-HKO mice were isolated and treated with PO for 12 h. The mRNA levels of \textit{Ppar\gamma} and its targets were also upregulated (Fig. 7E), together with the protein level of PPAR\gamma, in the IRF6-HKO group compared to the IRF6-Flox group (Fig. 7F). Next, we tested the mRNA levels of \textit{PPAR\gamma} and its targets in L02 cells. As expected, IRF6-KO upregulated the mRNA levels of \textit{PPAR\gamma} and its targets, as well as the protein levels of PPAR\gamma (Fig. 7G, H). In addition, \textit{PPAR\gamma} and its targets mRNA level were downregulated in the IRF6-overexpression L02 cell line (Fig. 7I). Consistent with these results, the protein level of PPAR\gamma decreased in the IRF6-overexpression L02 cell line (Fig. 7J). To further determine the relationship between IRF6 and PPAR\gamma in hepatic steatosis, we quantified the mRNA levels of \textit{Irf6} and \textit{Ppar\gamma} in mice livers after 24 w of HFD feeding. As expected, upregulated expression of \textit{Ppar\gamma} in the fatty liver was accompanied by decreased \textit{Irf6} expression (Fig. 7K). The same phenomenon was also demonstrated in \textit{ob/ob} and \textit{db/db} mice (Supporting Fig. S7) (23, 27).

Thus, IRF6 functions as a transcriptional regulator of \textit{Ppar\gamma}, and the downregulation of \textit{Irf6} is sufficient to increase mRNA and protein expression of \textit{Ppar\gamma} and its targets in hepatocytes. In other words, there are strong negative correlations between the expression levels of \textit{Irf6} and \textit{Ppar\gamma} in NAFLD.

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IRF6 suppresses hepatic steatosis via a PPARγ-dependent mechanism

To test the hypothesis that IRF6 inhibits the PPARγ gene and its associated metabolic disorders in hepatocytes, for clearer effect of PPARγ in hepatocytes, we generated a PPARγ knockout cell line in L02 cells firstly then transfected with an IRF6 small-interfering (si) RNA (si-IRF6) or a control siRNA (si-NC). Representative Western blot showed the expression of PPARγ and IRF6, and IRF6 was sufficiently attenuated by si-IRF6 transfection (Fig. 8A). Oil red O staining demonstrated that IRF6 deficiency aggravated PO-induced lipid accumulation in L02 cells. This effect was completely abolished by PPARγ depletion (Fig. 8B). Consistent results were observed in primary hepatocytes isolated from IRF6-Flox and IRF6-HKO mice. Co-treatment with the PPARγ inhibitor GW9662 partially alleviated PO-induced lipid accumulation (Fig. 8C) (28). Owing to the function of PPARγ on promoting lipid synthesis and accumulation in hepatocyte, we firmly believe that PPARγ could further reverse IRF6-induced fatty liver in vivo (24). Collectively, these data indicated that IRF6 inhibits lipid accumulation partially by repressing PPARγ-mediated mechanisms.

Discussion

In the present study, we identified the transcription factor IRF6 in hepatocytes as a critical regulator of liver steatosis. Hepatic Irf6 was suppressed by promoter hypermethylation during metabolic challenge. Further, deleting IRF6 in hepatocytes exacerbated HFD-induced steatosis in the murine liver, whereas the overexpression of IRF6 in hepatocytes attenuated HFD-induced hepatic lipid accumulation. Mechanistic studies demonstrated that IRF6 directly
binds to the transcription region of the \textit{Ppar\gamma} gene and subsequently halts the transcription of \textit{Ppar\gamma} and its target genes under physiological conditions. The insufficient production of \textit{Irf6} under HFD conditions failed to inhibit \textit{Ppar\gamma} and its targets, driving abnormalities in lipid metabolism. Thus, this study provides the first evidence of an essential function for IRF6 in hepatic steatosis.

IRF6 belongs to a family of nine transcription factors that share a highly conserved helix-turn-helix DNA-binding domain and a less conserved protein-binding domain. The function of IRF6 was unclear until the discovery of its determinant role in orofacial development in 2002. Various mutations in the \textit{IRF6} gene contribute to Van der Woude's or popliteal pterygium syndromes in humans, which manifest as cleft lip, cleft palate or skin and genital anomalies (29, 30). In the current study, we show that IRF6 is a beneficial mediator in the pathogenesis of liver steatosis, and its expression is downregulated by promoter hypermethylation. Conventionally, the majority of IRFs mediate IFN expression in response to viral invasion. However, evidence for the effect of IRF6 on IFNs is scant. To identify the transcriptional target of the IRF6 protein, ChIP-Seq and ChIP assays were applied. Our data show that IRF6 directly binds to the promoter of the \textit{PPAR\gamma} gene and inhibits its transcription in hepatocytes. Two previous reports also showed that IRF6 regulates \textit{Ppar\gamma} gene in macrophages and endothelial cells (31, 32). These studies, combined with our findings, suggest a more generalized transcriptional target gene for IRF6; however, we found a novel function of IRF6 in regulating different disease from the current report with different molecular mechanism. Moreover, each IRF member may have a wide spectrum of direct target genes depending on the disease setting (33). Serial studies of our group demonstrated, in addition
to directly regulating INF-α upon viral infection, hepatic IRF3 binds both the kinase domain of IKKβ and the promoter region of stearoyl–coenzyme A (CoA) desaturase 1 (SCD1), attenuating lipid accumulation and IR in the liver (34). IRF7 strongly promotes lipid accumulation, inflammation and insulin resistance in the liver (35). IRF9 interacts and promotes Ppar-α transcription to ameliorate hepatic steatosis or suppresses the activity of SIRT1 to ameliorate liver ischemic injury (36, 37). The above evidence suggests that IRFs may be potential molecular targets for the management of multiple diseases, but the cell type-specific roles of IRFs and the genetic modification signatures that trigger their activities must be identified during disease progression. In this scenario, the regulation of the promoter methylation status of IRF6 could be a promising strategy for the management of NAFLD.

PPARγ is a member of the nuclear hormone receptor superfamily that has been shown to be involved in the regulation of glucose homeostasis, adipogenesis, and inflammation (38, 39). However, there is an apparent paradox concerning the role of PPARγ in NAFLD. Numerous studies have shown that hepatic PPARγ functions as a steatogenic-inducer gene that activates de novo lipogenesis (23, 24, 27, 40) and increases hepatic triglyceride accumulation (41-44), although both short- and long-term administration of thiazolidinedione (TZD) and PPARγ agonists have been shown to improve hepatic steatosis in patients with NASH(45, 46). This discrepancy may suggest that TZD treatment improves hepatic steatosis because its primary insulin-sensitizing effect on adipose tissue outweighs its steatogenic effects in the liver (46-49). Thus, PPARγ antagonists may also not have an ideal therapeutic effect on IRF6 deficiency induced fatty liver in vivo, but specifically inhibiting hepatic PPARγ is a promising strategy for NAFLD therapy. Notably, in the current study, we found that...
increased IRF6 expression in hepatocytes is sufficient to suppress PPARγ gene expression and hepatic lipid accumulation and has a favorable effect on insulin resistance in HFD-fed animals. Thus, in terms of treating HFD-induced NAFLD, we propose that targeting hepatic IRF6 could be a more appropriate approach by upregulating its hepatic expression, which need to further study.

In conclusion, our results provide support for the proposed mechanism depicted in Fig. 8D. HFD-induced Irf6 promoter hypermethylation leads to the suppression of IRF6 gene transcription in hepatocytes. IRF6 functions as a transcription factor that binds the Pparγ gene and attenuates its expression. The induction of hepatic steatosis occurs due to the insufficient production of IRF6, which leads to a failure to inhibit the expression of Pparγ and its regulated lipogenesis-related factors in HFD-fed mice. The findings also highlight the possibility of developing novel therapeutic strategies to attenuate hepatic IRF6-PPARγ-mediated liver steatosis.

**Figure legends**

**Fig. 1:** IRF6 expression is downregulated in the fatty liver. (A) Histogram indicating the differentially expressed genes (DEGs: red, upregulated genes; blue, downregulated genes) in the high-fat diet group relative to the chow diet controls from the Supporting Table S1 database in the RNA-Seq assay. DEGs defined from the pairwise comparisons were required to satisfy two selection criteria: a fold change larger than 1.5 and a corresponding adjusted P value less than 0.05. (B) Intersection analysis among six databases revealed 4 commonly
changed genes. (C) Heat maps generated using the mRNA expression levels of the four genes. (D) \( \text{Irf6} \) mRNA levels were measured in the mice liver at the indicated time points after normal diet control (NC) or HFD feeding using qPCR (n=5/group). (E) \( \text{Irf6} \) mRNA levels were measured in the mice liver at the indicated time points after NC or HFHC feeding using qPCR (n=5/group). (F) Liver tissue lysates from mice fed a HFD or NC for 24 w. IRF6 protein levels were subjected to Western blot analysis (n=5/group). (G) Liver tissue lysates from mice fed HFHC or NC for 16 w. IRF6 protein levels were subjected to Western blot analysis (n=5/group). (H) Representative images of immunohistochemical staining for IRF6 in liver sections from mice fed a HFD and NC are shown (n=3/group). (I) Representative images of immunohistochemical staining for IRF6 in liver sections from patients with normal and nonalcoholic fatty liver (normal: n=4/group; NAFLD: n=3/group). For all statistical plots, the data are presented as the mean ± s.d.; *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.

**Fig. 2:** Hepatic expression of \( \text{Irf6} \) is suppressed by promoter hypermethylation in HFD-fed mice. (A) Hepatocytes were co-treated with 0.5mM palmitate and 1.0mM oleic acid (PO) for 24 h. \( \text{Irf6} \) mRNA expression was measured by q-PCR (n=3), and (B) IRF6 protein expression was measured by Western blotting (n=3). (C) Hepatocytes were treated with PO for 24 h and adding protease inhibitor, MG132 (25 μM) for 12 h before sample collection. IRF6 protein was measured by Western blotting (n=3). (D) CpG island detected by Meth Primer software (top). Schematic of quantitative analysis of methylated alleles (QAMA) for the \( \text{Irf6} \) gene (bottom).
Non-methylated cytosine is converted to uracil and then thymine, but not methylated cytosine, by PCR, as indicated by the arrow. (E) The presence of 5-methyl cytosine was detected by DNA bisulfite sequencing in the normal (top) and fatty mice liver (bottom). The percentage of methylated cytosine is indicated on the left, and Irf6 mRNA expression is labeled on the right. Each column represents a CpG site (28 total) and each row represents a genomic DNA clone (n = 10). White blocks indicate an unmethylated CpG site, red blocks a methylated CpG site. (F) Primary hepatocytes were co-treated with PO and 10 μM 5-AzaC. Irf6 mRNA expression was quantified by qPCR (n=3), and (G) Irf6 protein levels were measured by Western blotting (n=3). For all statistical plots, the data are presented as the mean ± s.d.; *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.

**Fig. 3:** IRF6 prevents lipid accumulation in hepatocytes *in vitro*. (A) Representative immunofluorescence staining of normal mice liver sections is shown. The liver tissues were stained with an IRF6 antibody (red). Nucleus were stained with DAPI (blue). (n=3/group) (B) Cytosolic and nuclear fractions from primary hepatocytes were separated, and IRF6 expression was measured by Western blotting (n=3). GAPDH indicates the cytosol, and Lamin B indicates the nucleus. (C, E) An IRF6-overexpression cell line (C) and an IRF6-knockout cell line (E) were established in L02 cells. IRF6 protein expression was tested by Western blot analysis. (D) Representative photomicrographs with Oil Red O staining are shown for the IRF6-overexpression cell line with or without PO exposure. Oil red O stained area was quantified by image pro plus. (F) Representative photomicrographs with Oil Red O staining.
staining are shown for the *IRF6* knockout cell line with and without PO exposure. The Oil red O stained area was quantified by Image Pro Plus. (G) The *IRF6*-KO cell line or control cell line was treated with PO for 12 h. The indicated genes were measured using qPCR. For all statistical plots, the data are presented as the mean ± s.d.; *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.

**Fig. 4:** Hepatocytes-specific deletion of IRF6 (IRF6-HKO) aggravates HFD-induced hepatic steatosis. (A) Expression of IRF6 in liver, spleen, kidney, and heart samples from IRF6-Flox and IRF6-HKO mice. GAPDH served as a loading control. (B) Body weights of IRF6-HKO mice or their Flox controls after 0, 4, 8, 12, 16, 20 and 24 weeks of NC or HFD feeding (n=10/group). (C-E) Fasting blood glucose levels (C), fasting insulin levels (D), and HOMA-IR values (E) in NC-fed or HFD-fed IRF6-Flox or IRF6-HKO mice at the indicated time points (n=10/group). (F) Blood glucose levels after NC or HFD feeding for 23 continuous weeks in IRF6-Flox or IRF6-HKO mice during GTT assay. (G, H) LW and LW/BW ratios of IRF6-HKO mice and their corresponding littermate controls after NC or HFD feeding for 24 weeks (n=10/group). (I) Representative images of hematoxylin and eosin (upper) and Oil Red O (bottom) staining in liver sections from HFD-fed IRF6-Flox and IRF6-HKO mice (n=8/group). (J, K) Serum lipid content (J) and serum ALT and AST levels (K) in IRF6-Flox and IRF6-HKO mice fed an NC or HFD for 24 weeks (n=10/group). For all statistical plots, the data are presented as the mean ± s.d.. For figure B, C and F, *P<0.05; **P<0.01, IRF6-Flox HFD group compared with the IRF6-Flox NC group; *P<0.05, **P<0.01, IRF6-HKO HFD group.
compared with the IRF6-Flox HFD group. For others data *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.

**Fig. 5:** Hepatocytes-specific overexpression of IRF6 (IRF6-HTG) mitigates HFD-induced hepatic steatosis. (A) Expression of IRF6 in the livers of NTG and IRF6-HTG mice. β-actin served as a loading control. (B-E) Body weights (B), fasting blood glucose levels (C), fasting insulin levels (D), and HOMA-IR values (E) of IRF6-HTG mice and their littermate controls at the indicated time points in response to HFD feeding (n=8/group). (F) GTTs were performed in IRF6-NTG and IRF6-HTG mice after HFD feeding for 23 weeks, and the corresponding areas under the curves were calculated (n=8/group). (G, H) LW and LW/BW ratio in NTG and IRF6-HTG mice after 24 weeks of NC or HFD feeding (n=8/group). (I) Representative images of H&E-stained (upper) and Oil red O-stained (lower) liver sections from HFD-fed NTG and IRF6-HTG mice. (J, K) Serum lipid content (J) and serum ALT and AST levels (K) in NTG and IRF6-HTG mice fed a HFD or NC for 24 weeks (n=8/group). For all statistical plots, the data are presented as the mean ± s.d.. For figure B, C and F, *P<0.05; **P<0.01, IRF6-NTG HFD group compared with the IRF6-NTG NC group; #P<0.05, ##P<0.01, IRF6-HTG HFD group compared with the IRF6-NTG HFD group. For others data *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.
**Fig. 6** Transcriptional behavior governed by IRF6 during hepatic steatosis. (A) Schematic of the experimental strategy used to identify the potential target of IRF6 in liver steatosis. (B) Cluster images showing global sample distribution profiles and relationships analyzed by hierarchical clustering. (C) Volcano plots indicating the differentially expressed genes (DEGs: red, upregulated genes; blue, downregulated genes) in the IRF6-HKO group relative to IRF6-Flox controls fed a HFD for 10 weeks in the DGE assay. The vertical dashed gray lines in the plot represent log2 normalized fold changes of 0.585 and -0.585. The horizontal dashed gray line represents an adjusted P value of 0.05. (D) The top 5 enriched biological process contributing to IRF6 function determined by GO analysis based on the DEGs from the DGE assay (top). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the four identified lipid metabolism-related processes from the above GO analysis. In B-D, n=3 mice in each group for DGE. (E) Pie chart of the distribution of the IRF6 binding sites from ChIP-Seq. (F) Five genes with IRF6 binding from 3000 bp upstream to 3000 bp downstream of the TSS expressed differentially in RNA-Seq. Heat map (right) and ChIP-Seq information of the five genes are shown respectively.

**Fig. 7** IRF6 repressed PPARγ expression at the transcription level. (A) The IGV diagram showing the IRF6 binding position near the TSS of PPARγ gene from ChIP-Seq data performed in the 3xFlag-IRF6 L02 cell line. (B) The independent ChIP assay was performed in the 3xFlag-IRF6 L02 cell line to confirm IRF6 binding to the PPARγ promoter. A control genomic region on the same chromosome was also amplified (Chr3 ctr). P1 indicates the
primer designed inside the \textit{PPAR}_{\gamma} peak, and P2 indicates the primer designed outside the \textit{PPAR}_{\gamma} peak as showed in Fig. 7A. (C) Analysis of the genes affected by PPAR signaling. PPAR target genes and their cellular functions are indicated. Only the differentially expressed genes were colored according the scale of fold change. (D) The mRNA levels of PPAR target genes in the IRF6-HKO mice liver compared to the IRF6-FLOX, as described in (C). (E) Representative \textit{Ppar} and targets mRNA levels in isolated IRF6-HKO or IRF6-Flox primary hepatocytes that were treated with PO for 12 h. (F) The protein expression level of PPAR\textgamma{} in IRF6-HKO primary hepatocytes, as described in (E), β-actin was used as the loading control. (G) The relative mRNA expression of \textit{PPAR}_{\gamma} and its targets in IRF6-KO or wild-type (WT) cell lines that were treated with PO for 12 h. (H) The protein level of PPAR\textgamma{} in the L02 cell line, as described in (G). (I) Representative mRNA levels of \textit{PPAR}_{\gamma} and its targets in IRF6 or vector overexpression L02 cell lines treated with the same treatment described above. (J) The protein level of PPAR\textgamma{}, as described in (I). (K) \textit{Irf6} and \textit{Ppar} mRNA expression levels measured in the liver in mice fed a HFD for 24 w (n=4). For all statistical plots, the data are presented as the mean ± s.d.; *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.

\textbf{Fig. 8} IRF6 suppresses hepatic steatosis via a \textit{PPAR}_{\gamma}-dependent mechanism. (A) \textit{PPAR}_{\gamma}-KO and WT cell lines in L02 cells were transfected with si-\textit{IRF6} or si-NC for 36 h followed by PO treatment for 24 h. Western blot of PPAR\textgamma{} and IRF6 protein levels. β-actin was used as a loading control. (B) Representative photomicrographs of Oil Red O staining.
with treatments, as described in (A) (left), and quantification of the area of Oil red O staining per cell by ImagePro Plus (right) (n=3). (C) Primary hepatocytes were isolated from IRF6-Flox and IRF6-HKO mice, treated with bovine serum albumin (BSA) or PO combined with DMSO or GW9662 (10 μM) for 24 h, and stained with Oil red O (left), and the area of Oil red O staining per cell was quantified (right) (n=3). (D) Schematic diagram of the proposed mechanism. For all statistical plots, the data are presented as the mean ± s.d.; *p<0.05, **p<0.01 and n.s. indicates no significance between the two indicated groups.

Reference:


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Figure 3

A

IRF6  
DAPI  
Merge

B

Primary hepatocyte

IRF6  
GAPDH  
Lamin B

C

LO2

IRF6

IRF6

β-actin

D

Vector

IRF6

IRF6

β-actin

E

LO2

WT

IRF6-KO

IRF6

IRF6

β-actin

F

WT

IRF6-KO-1

IRF6-KO-2

Oil red O positive area

Oil red O positive area

G

WT PO  IRF6-KO PO

Relative mRNA levels

Cholesterol synthesis
Lipogenesis
Fatty acid uptake
Fatty acid oxidation

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Figure 4

A

Liver Spleen Kidney Heart
Flx HKO Flx HKO Flx HKO Flx HKO
IRGF GAPDH

B

Flx-NC Flx-HFD
HKO-NC HKO-HFD

C

Flx-NC Flx-HFD
HKO-NC HKO-HFD

D

Flx-NC Flx-HFD
HKO-NC HKO-HFD

E

24W

F

Blood glucose (mmol/L)

G

15

H

Liver weight (%)

J

Liver triglycerides (mg/g)

K

ALC (U/L)

n.s.

Ox Red 0

n.s.

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