Original Article

MOLECULAR METABOLISM

IGFBP2 functions as an endogenous protector against hepatic steatosis via suppression of the EGFR-STAT3 pathway

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ABSTRACT

Objective: Non-alcoholic fatty liver disease (NAFLD) is deemed as an emerging global epidemic, whereas the underlying pathogenic mechanism remains to be clarified. We aimed to systemically analyze all the NAFLD-related gene expression datasets from published human-based studies, by which exploring potential key factors and mechanisms accounting for the pathogenesis of NAFLD.

Methods: Robust rank aggregation (RRA) method was used to integrate NAFLD-related gene expression datasets. For fatty liver study, adeno-associated virus (AAV) delivery and genetic knockout mice were used to create IGFBP2 (Insulin-like growth factor binding protein 2) gain- or loss-of function models. Western blot, Co-immunoprecipitation (Co-IP), immunofluorescent (IF) staining, luciferase assay, molecular docking simulation were performed to reveal the IGFBP2-EGFR-STAT3 axis involved. Key axis protein levels in livers from healthy donors and patients with NAFLD were assessed via immunohistochemical staining.

Results: By using RRA method, the present study identified IGFBP2 being the most significantly down-regulated gene in all NAFLD subjects. The decreased IGFBP2 expression was further confirmed in the liver tissues from patients and animal models of NAFLD. IGFBP2 deficiency aggravated hepatic steatosis and NASH phenotypes and promoted lipogenic gene expression both *in vivo* and *in vitro*. Mechanistically, IGFBP2 directly binds to and regulates EGFR, whereas blockage of the IGFBP2-EGFR complex by knockdown of IGFBP2 resulted in the EGFR-STAT3 pathway activation, which in turn promoted the promoter activity of *Srebf1*. By using molecular docking simulation and protein-protein interaction analysis, the sequence of 233-257 amino acids in IGFBP2 was characterized as a key motif responding for its specific binding to EGFR and the protective effect against hepatic steatosis.

Conclusions: The current study has, for the first time, identified IGFBP2 as a novel protector against hepatosteatosis. The protective effect is mediated by its specific interaction with EGFR and thereby suppressing the EGFR-STAT3 pathway. Therefore, pharmaceutically targeting the IGFBP2-EGFR-STAT3 axis may provide a theoretical basis for for the treatment of NAFLD/NASH and the associated diseases.

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Keywords Non-alcoholic fatty liver disease; Robust rank aggregation; Insulin-like growth factor binding protein 2; Hepatic steatosis; Epidermal growth factor receptor; Signal transducer and activator of transcription 3

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been emerging an epidemic over the world, affecting nearly 25% of the global adults [1] and even up to 10% of children in developed countries [2]. NAFLD embodies a spectrum of liver disease from simple steatosis progressing through nonalcoholic steatohepatitis (NASH) to fibrosis, cirrhosis and end-stage liver failure [3]. Besides, NAFLD is often accompanied with other metabolic diseases, such as dyslipidemia, hypertension, obesity and type 2 diabetes mellitus (T2DM), and may leads to hepatocellular carcinoma (HCC) [4]. However, the pathogenesis and progression of the disease is not completely understood yet. As such, there is still a lack of effective way to prevention and treatment of NAFLD and its progression.

In hepatocytes, lipid homeostasis is balanced between lipogenesis/fatty acid uptake and fatty acid oxidation/lipoprotein secretion [5]. Hepatic steatosis is a hallmark of NAFLD that represents a condition of excess triglyceride accumulation in hepatocytes [6]. Previous studies have

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Abbreviations

non-alcoholic fatty liver disease (NAFLD)
non-alcoholic fatty liver (NAFL)
non-alcoholic steatohepatitis (NASH)
type 2 diabetes mellitus (T2DM)
hepatocellular carcinoma (HCC)
de novo lipogenesis (DNL)
acetyl-CoA carboxylase (ACC)
fatty acid synthase (FASN)
stearoyl-CoA desaturase 1 (SCD1)
sterol regulatory element-binding transcription protein 1 (SREBP1)
epidermal growth factor receptor (EGFR)
tyrosine kinase inhibitor (TKI)
hepatic stellate cell (HSC)
Robust rank aggregation (RRA)
insulin-like growth factor binding protein 2 (IGFBP2)
Gene Expression Omnibus (GEO)
differentially expressed genes (DEGs)
gene ontology (GO)
Kyoto Encyclopedia of Genes and Genomes (KEGG)
high-fat diet (HFD)
Intraperitoneal glucose tolerance tests (IPGTT)
intraperitoneal insulin tolerance tests (IPITT)
glycosylated serum protein (GSP)
aspartate aminotransferase (AST)
alanine aminotransferase (ALT)
free fatty acids (FFA)
hematoxylin and eosin (H&E)
bovine serum albumin (BSA)
immunohistochemical (IHC)
Oil Red O (ORO) staining
poly(ADP-ribose)-polymerase (PARP)
metabolic syndrome (MetS)
body mass index (BMI)
heparin-binding domain (HBD)
nuclear localization signal (NLS)
fast-food diet (FFD)

shown that hepatic *de novo* lipogenesis (DNL) drives fatty acid synthesis and contributes to about 5% of the triglyceride deposition in the livers of healthy individuals, whereas up to 20%—25% in the livers of NAFLD subjects [7,8]. Therefore, the key regulators of hepatic DNL, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1) and sterol regulatory element-binding transcription protein 1 (SREBP1), are expected to be promising therapeutic targets for NAFLD [9]. Another promising candidate for NAFLD treatment proposed recently is epidermal growth factor receptor (EGFR) that is highly expressed in the liver and functions as a receptor tyrosine kinase involved in a wide variety of biological processes [10]. Elevated hepatic expression of EGFR existed in NAFLD/NASH patients and highly associated with the disease severity [11]. Experimental evidence showed that inhibition of EGFR by a tyrosine kinase inhibitor (TKI) or a kinase domain mutation significantly alleviated hepatic steatosis [12—14]. Moreover, the EGFR inhibitor treatment suppressed hepatic stellate cell (HSC) activation and attenuated fibrosis and NASH phenotypes in animal models [13,15], suggesting a key pathogenic role of EGFR. However, the molecular mechanism underlying the effect of EGFR in NAFLD/NASH remains to be identified.

Owing to significant advantages in biomedical research, microarray and high-throughput transcriptome sequencing have been widely used in numerous research fields, including NAFLD. Nonetheless, the research findings derived from these technologies are somewhere inconsistent or even contradictory, which may attribute to various factors, including the sample size, sample sources and the differences in study platforms. Integrated analysis based on multiple datasets has been proven to greatly reduce the influence of various confounding factors and provide more stable and reliable results [16]. Robust rank aggregation (RRA) is an efficient means of deep detection and mining of transcriptomic data, which has four key features: strong robustness to noise, ability to deal with incomplete ranking, giving significant scores to each element in the result ranking, and high computational efficiency [17]. Based on this powerful systematic integrative analysis, we are able to merge the availability of multiple experimental datasets, the continued progress in developing technological platforms, and various statistical methods of analyzing data. In the present study, we have searched for all published datasets related to human NAFLD transcriptomic studies in the GEO and ArrayExpress databases, which currently dominated in the international bioinformatics scope. By using the RAA method to integrate all these available human fatty liver transcriptome datasets, we identified insulin-like growth factor-binding protein 2 (IGFBP2) as the most significantly differentially expressed gene (DEG) in NAFLD patients. Intriguingly, IGFBP2 has been regarded as a hepatokine, playing an important role in body metabolism and closely associated with metabolic diseases, such as obesity and diabetes [18,19]. More recently, IGFBP2 has been suggested to be involved in the pathogenesis of NAFLD/NASH [20,21]. As a secreted protein, IGFBP2 is one of the six IGFBPs circulating in plasma and functions chiefly through its binding to IGF-1/2 [22,23]. Moreover, existing evidence has demonstrated multiple effects of IGFBP2 independent on IGF-1/2. For instance, IGFBP2 is co-expressed and interacts with EGFR and regulates its downstream signaling pathways in glioma and melanoma cells [24,25]. In this study, we provided both experimental evidence and mechanistic data uncovering that IGFBP2 functions as an endogenous protector against NAFLD via its interaction with EGFR and selective suppression of the downstream STAT pathway in hepatocytes. Thus, not only our study identified a novel molecular mechanism that involved in the pathogenesis of NAFLD, but may also provide a potential intervention target for the treatment of NAFLD/NASH.

2. METHODS

Detailed in Supplementary materials.

3. RESULTS

3.1. Hepatic IGFBP2 expression is decreased in NAFLD subjects

By accessing to human GEO database, we included GSE48452, GSE63067, GSE72756, GSE105127, GSE89632, GSE107231, GSE61260, and GSE126848 datasets in the study, totally containing 111 healthy controls and 89 NAFLD subjects with simple steatosis (Supplementary Table 1). The RRA approach was adopted to integrate multiple datasets and identify robust DEGs. There was a total of 129 significant DEGs (with 64 down-regulated and 65 up-regulated) in the integrated analysis. The heatmap of top 20 down- and up-regulated genes was shown in Supplementary Figure 1. Among them, the top 5 significantly up-regulated genes were CYP7A1 (P = 6.15E-15), TMEM154 (P = 2.21E-14), FNDC5 (P = 2.62E-11), PEG10 (P = 4.76E-11), and CLGN (P = 1.01e-08), the top 5 genes with significantly down-regulated expression were IGFBP2 (P = 2.60E-17),

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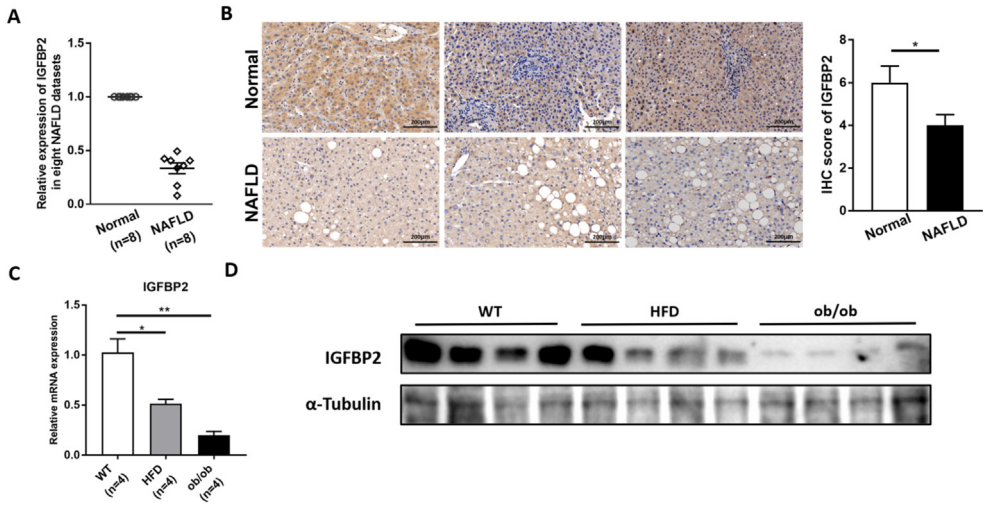

Figure 1: Hepatic expression of IGFBP2 is decreased in NAFLD patients and experimental mice. (A) Relative expression levels of IGFBP2 in normal and NAFLD subjects collected from 8 human NAFLD datasets. (B) IHC staining (left) and its quantification (right) for IGFBP2 expression in the liver sections from normal controls and NAFLD patients (n = 8/group). (C) The mRNA and (D) protein expression levels of IGFBP2 in control, HFD-fed and ob/ob mice (n = 4/group). $^{*}P < 0.05$, $^{**}P < 0.01$.

P4HA1 ($P = 3.60E-10$), CXCL13 ($P = 3.81E-10$), ABCB11 ($P = 2.64E-09$) and RND1 ($P = 4.96E-09$). Remarkably, IGFBP2 was the most significant gene in the analysis, showing a consistent down-regulation in NAFLD subjects from all these eight datasets (Figure 1A).

We further performed the gene ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the aberrant expressed genes derived from RRA. The results from term of biological process showed that regulation of lipid metabolic process (GO: 0019216, $P = 4.56E-06$) and regulation of insulin-like growth factor receptor signaling pathway (GO: 0043567, $P = 1.31E-06$) were most enriched in the up-regulated and down-regulated genes, respectively. Subsequent KEGG analysis showed that alpha-linolenic acid metabolism (hsa00592, $P = 0.003$) and Jak-STAT signaling pathway (hsa04630, $P = 0.004$) were mostly associated with the up-regulated and down-regulated genes, respectively (Supplementary Figure 2, 3).

To further confirm the down-regulation of IGFBP2 in NAFLD, we evaluated IGFBP2 expression in human liver samples by IHC staining from randomly collected 8 male NAFLD patients and 8 controls with matched age, gender and BMI. Compared with normal controls, expression levels of IGFBP2 were substantially decreased in the livers of NAFLD patients (Figure 1B). In line with the findings in humans, both mRNA and protein levels of IGFBP2 were markedly down-regulated in the livers isolated from either HFD-induced obese (DIO) or leptin-deficient *ob*/*ob* mice, both represent a common feature of human NAFLD (Figure 1C,D).

3.2. IGFBP2 deletion aggravates NAFLD and NASH progression in DIO mice

To investigate whether IGFBP2 actively impact the progression of NAFLD, *Igfbp2*-knockout (*Igfbp2*-KO) mice were generated and subjected to a DIO model by feeding an HFD for 8 weeks. Fed with 8-weeks chow diet, *Igfbp2*-KO and control mice did not demonstrate any obvious steatotic phenotype (Supplementary Figure 4E—I). In line with previous reports [26], after 8-weeks HFD feeding, WT mice exhibited a mild steatotic phenotype in the liver as shown in Figure 2A. Remarkably, as compared with WT, *Igfbp2*-KO mice were significantly impaired from diet-induced hepatic steatosis as indicated by more severe lipid infiltration in the livers determined by H&E and ORO staining (Figure 2A), together with higher levels of TG, TC and free fatty acid (FFA) in both liver and serum (Figure 2B—E). In addition, liver weights, body weights and their ratios were also increased in *Igfbp2*-KO mice, whereas they had similar daily HFD intake, compared with WT mice (Supplementary Figure 4A—D). Moreover, serum ALT and AST activities were significant higher in *Igfbp2*-KO mice than the controls, suggesting more damages in the IGFBP2 deficient liver (Figure 2F,G). Notably, *Igfbp2*-KO mice exhibited higher fasting blood glucose, glycosylated serum protein (GSP) and insulin levels (Figure 2H—J), and impaired insulin sensitivity as reflected in worse glucose and insulin tolerance (Figure 2K,L). To exclude the systemic effects on hepatic steatosis in *Igfbp2*-KO mice, we generated the IGFBP2 liver-specific knockout (LKO) mice, which were also fed with HFD for 8 weeks. Similarly, IGFBP2 LKO significantly promoted the hepatic lipid accumulation showed by H&E and ORO staining and enhanced the hepatic TG content (Figure 6D,G).

Given the significant effect of IGFBP2 deficiency on hepatic steatosis, we investigated its effect in a long-term model of DIO by extending HFD feeding from 8 to 20 weeks. Similar with the short-term DIO models, *Igfbp2*-KO mice displayed more heavy hepatic lipid deposition than WT controls, as determined by ORO and H&E staining (Figure 2M) and hepatic contents of TG, TC and FFAs, after 20-weeks HFD feeding (Supplementary Figure 5A—C). Accordingly, the DIO associated metabolic dysfunctions, including elevated serum lipids, fasting blood glucose and impaired insulin sensitivity, were also more severe in *Igfbp2*-KO mice than those in WT controls after 20-weeks HFD feeding (Supplementary Figure 5D—H). Furthermore, while compared with 8 weeks HFD feeding, serum levels of ALT and AST were markedly increased in both WT and *Igfbp2*-KO mice following 20-weeks HFD feeding, more serious liver injury was observed in *Igfbp2*-KO mice as they had much higher levels of ALT and AST than that in WT mice (Figure 2O,P). Moreover, IGFBP2 deficiency increased hepatic fibrosis as revealed by the Masson and sirius red staining (Figure 2N), and promoted hepatocyte apoptosis as shown by TUNEL staining (Figure 2Q) and elevated levels of poly (ADP-ribose) polymerase (PARP) cleavage (Supplementary Figure 7A). In addition, a higher abundance of F4/80-positive macrophages was observed in *Igfbp2*-KO mice, compared to WT mice (Figure 2Q). Correspondingly, the mRNA levels of

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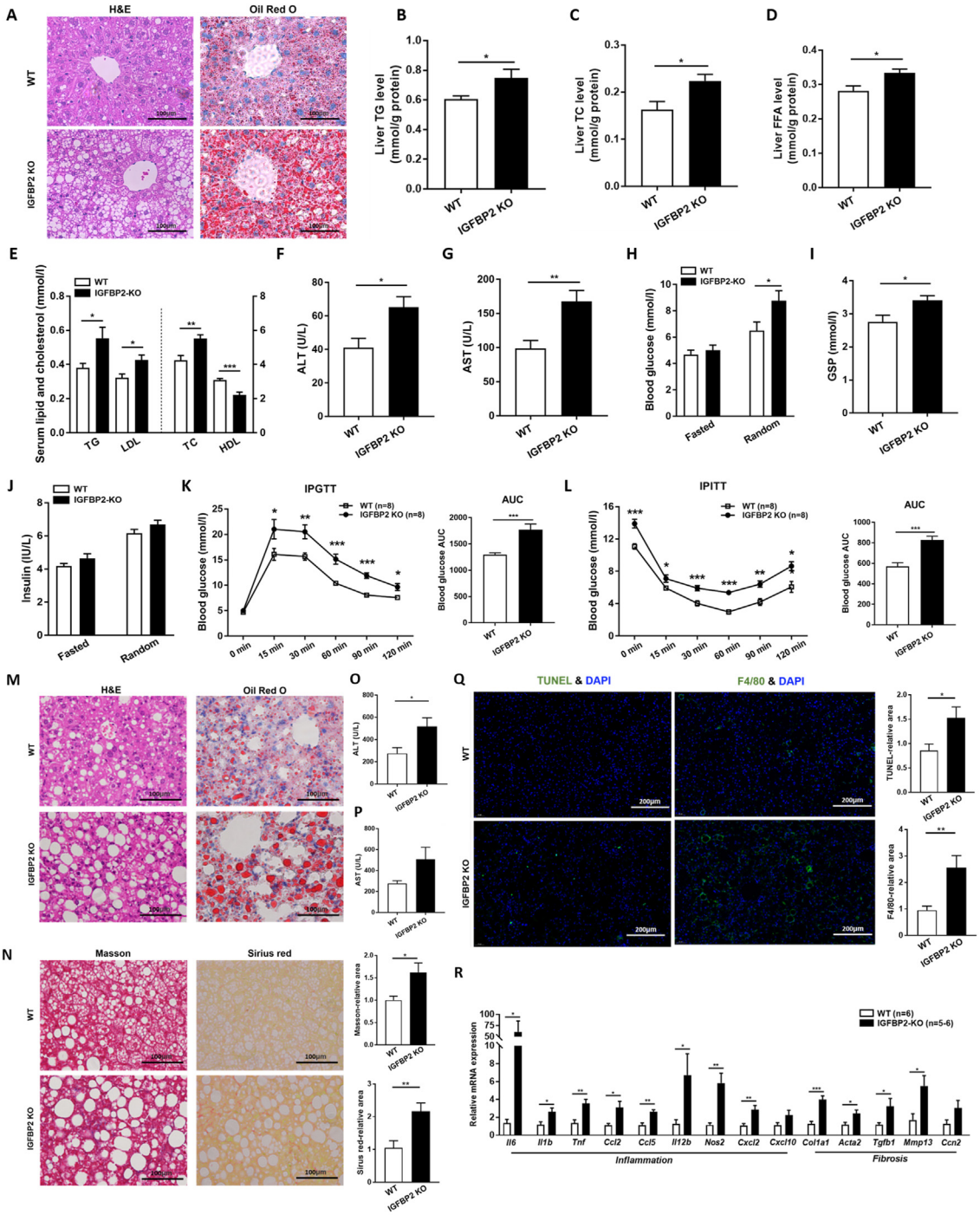

Figure 2: IGFBP2 depletion aggravates NAFLD/NASH and metabolic dysfunction in DIO mice. (A–L) WT and *Igfbp2*-KO mice (n = 8/group) were fed with HFD for 8 weeks. (A) Representative images of Oil Red O and H&E staining of the liver sections from each group. Scale bar, 100 μm. (B) The hepatic content of TG, (C) TC and (D) FFA, (E) serum TG, TC, LDL-c, and HDL-c levels, (F) serum ALT and (G) AST activities, (H) levels of fasted and random blood glucose, (J) insulin and (I) glycosylated serum proteins, (K) IPGTT and (L) IPITT were determined in WT and *Igfbp2*-KO mice (n = 8/group). (M–R) WT and *Igfbp2*-KO mice (n = 6/group) were fed with HFD for 20 weeks. Representative images of (M) H&E and Oil Red O staining, (N) Masson and Sirius red staining, and (Q) TUNEL and F4/80 immunofluorescence staining of the liver sections from WT and *Igfbp2*-KO mice. (O) Serum ALT and (P) AST activities were measured. (R) Quantitative PCR analysis of mRNA expression levels of the genes involved in inflammation and fibrosis in the livers from each group (n = 5–6/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pro-fibrotic and pro-inflammatory genes were significantly upregulated in the livers of *Igfbp2*-KO mice (Figure 2R). Taken together, these data indicate an important protective role of IGFBP2 in both the development of NAFLD and its progression to NASH.

In an attempt to understand the protective effect of IGFBP2 against hepatic steatosis in transcriptional levels, we conducted a transcriptomic analysis in the liver isolated from two randomly-selected *Igfbp2*-KO and WT mice. Totally, 688 significant DEGs (516 down-

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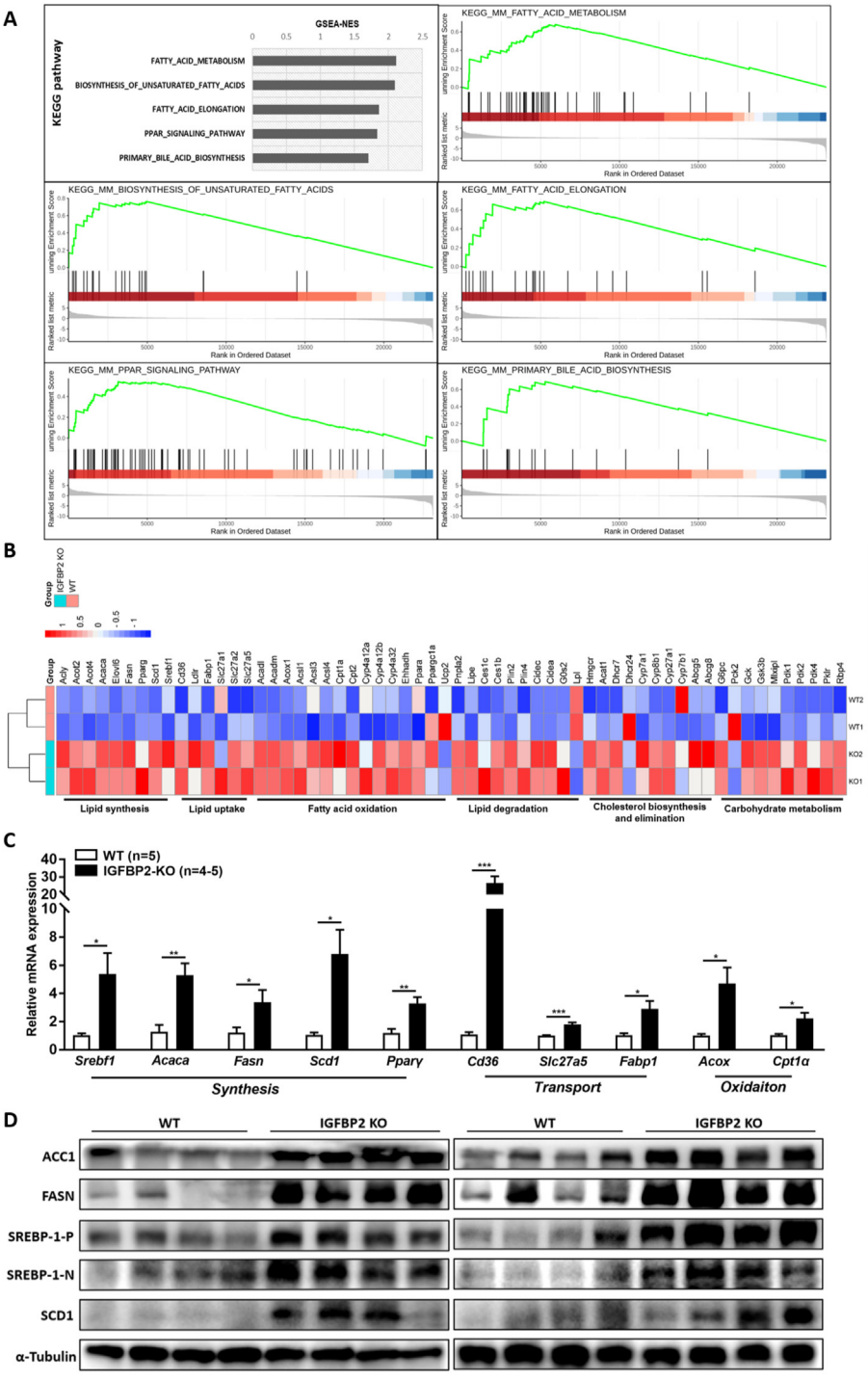

Figure 3: IGFBP2 depletion results in transcriptomic alterations related to NAFLD progression. (A) GSEA analysis shows the enriched KEGG pathways in *Igfbp2*-KO mice compared with the controls. (B) Heatmap shows the mRNA expression of the signature genes involved in lipid and glucose metabolism in the livers of WT and *Igfbp2*-KO mice (n = 2/group). (C) Quantitative PCR analysis of mRNA expression of genes involved in lipid metabolism in the livers of WT and *Igfbp2*-KO mice (n = 4–5/group). (D) Western blots showing protein expression levels of genes involved in *de novo* lipogenesis in the livers for each group (n = 8/group). (D) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

regulated and 172 up-regulated) were determined between *Igfbp2*-KO and WT group (Supplementary Figure 6A). GO analysis showed that the most DEGs were enriched in the biological processes related to NAFLD, including redox reaction and lipid metabolism (Supplementary Figure 6B). Correspondingly, GSEA showed that the pathways related to biosynthesis, elongation and metabolism of fatty acid and PPAR signaling were enriched in *Igfbp2*-KO mice (Figure 3A). Heatmap of the transcriptomic analysis displayed heightened hepatic expression of the genes related to lipid synthesis, transport and degradation and fatty acid oxidation, as well as cholesterol and carbohydrate metabolism in *Igfbp2*-KO mice (Figure 3B). To further confirm the findings from transcriptomic analysis, we examined both mRNA and protein

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levels of the key molecules involved in lipogenic program, including SREBP1, ACC1, FASN, and SCD1. All of these genes were significantly up-regulated in the IGFBP2 deficient livers (Figure 3C,D).

3.3. IGFBP2 deficiency facilitates hepatosteatosis *in vitro*

In order to investigate the effect of IGFBP2 directly on hepatocytes, we used HepG2 and Huh7, the two human hepatocellular cancer cell lines that maintain normal feature of lipid metabolism and were commonly used for investigation of hepatic steatosis through palmitic acid (PA) stimulation [27,28]. In line with the aforementioned findings *in vivo*, the lentivirus-shRNA-mediated IGFBP2 knockdown significantly increased lipid accumulation in both HepG2 and Huh7 cells, as indicated by ORO staining and intracellular TG levels (Supplementary Figure 8A, B). Moreover, IGFBP2 knockdown significantly enhanced expression of genes for the programs of DNL, lipid transportation, gluconeogenesis and inflammation, while reduced expression of the genes involved in lipolysis and proliferation (Supplementary Figure 9A—B, D). Furthermore, similar findings were observed in the primary hepatocytes isolated from WT and *Igfbp2*-KO mice (Supplementary Figure 8C and 9C), further indicating a protective effect of IGFBP2 on hepatocytes against steatosis.

3.4. IGFBP2 interacts with EGFR and regulates the EGFR-STAT3 pathway

Subsequently, we aimed to explore the underlying mechanisms responsible for IGFBP2 protection against hepatic steatosis. Because emerging evidence has revealed the IGF-independent functions of IGFBP2 involved in multiple intracellular and nuclear signaling networks, we investigated its effect on the EGFR pathway [24,25]. Interestingly, knockdown of IGFBP2 profoundly promoted EGF-induced activation of EGFR and the downstream STAT3 phosphorylation, without obvious effect on the Akt and ERK signaling arms in hepatocytes (Figure 4A). The activation of EGFR-STAT3 pathway was also observed in *Igfbp2*-KO mice (Supplementary Figure 10A). Moreover, pretreatment of erlotinib, a specific EGFR TKI, completely inhibited the active EGFR and STAT3 induced by IGFBP2 knockdown (Figure 4B,C), suggesting a specific role of IGFBP2 in potentiating EGFR-STAT3 pathway. To elucidate how IGFBP2 regulates the EGFR-STAT3 pathway, we firstly assessed whether IGFBP2 can physically interacts with EGFR. Evidently, IF staining of either Huh7 or HepG2 cells showed co-localization of the endogenous EGFR and IGFBP2 proteins in cytoplasm and cell membrane (Figure 5A). Furthermore, Co-IP studies revealed that EGFR was co-precipitated with IGFBP2, further indicating a direct interaction between these two endogenous proteins existed in hepatocytes under the basal conditions (Figure 5B and Supplementary Figure 10B). Of note, the amount of IGFBP2 proteins binding to EGFR was significantly reduced following EGF or PA treatment (Figure 5C,D), suggesting a dynamic EGFR-IGFBP2 interaction in hepatocytes.

3.5. EGFR exacerbates hepatosteatosis through the STAT3 pathway

We then asked whether EGFR accounts for the effect of IGFBP2 on hepatic steatosis. For addressing this question, we firstly defined if EGFR activity regulates lipid metabolism in hepatocytes. Remarkably, treatment of hepatocytes with EGF resulted in a significant increase in

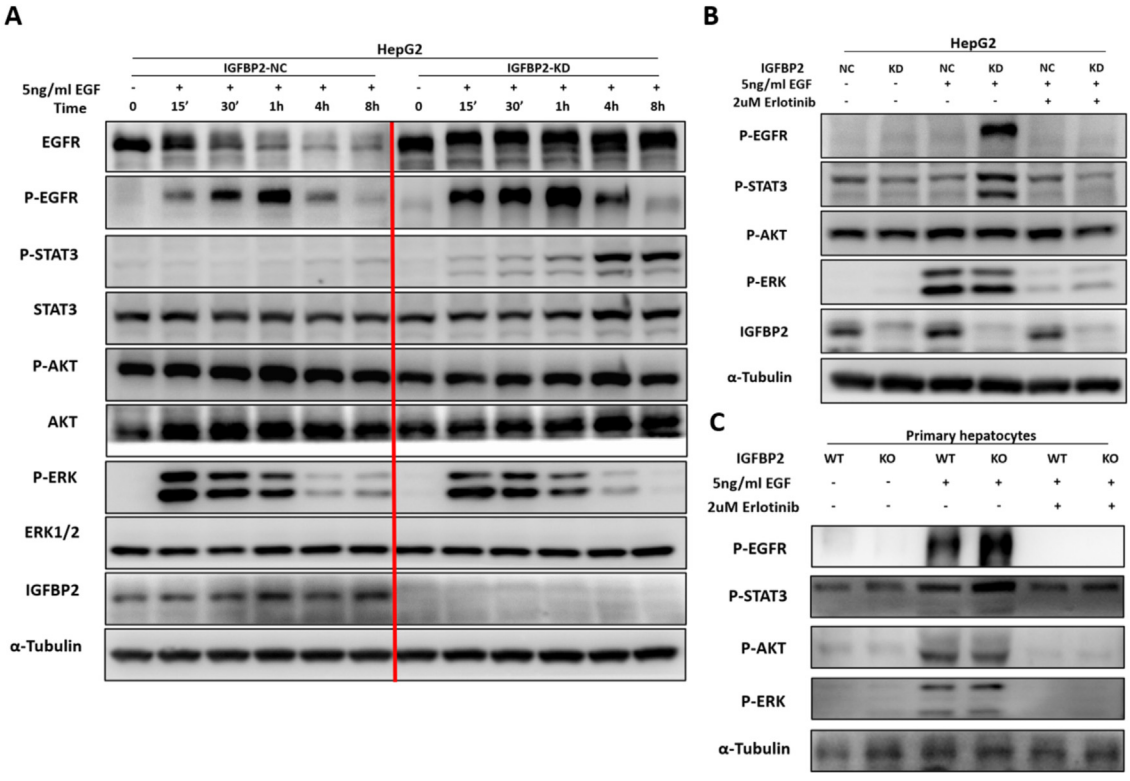

Figure 4: IGFBP2 knockdown promotes activation of the EGFR-STAT3 pathway in hepatocytes. (A) Immunoblot analysis of HepG2 cells infected with lentivirus-sh-NC or lentivirus-sh-IGFBP2 and treated with 5 ng/ml EGF for different periods. (B) Immunoblot analysis of HepG2 cells infected with lentivirus-sh-NC or lentivirus-sh-IGFBP2 which were pre-treated with vehicles alone or 2 μM erlotinib for 1 h, and then treated with 5 ng/ml EGF for 15 min. (C) Immunoblot analysis of primary hepatocytes isolated from WT and *Igfbp2*-KO which were pre-treated with vehicles alone or 2 μM erlotinib for 1 h, and then treated with 5 ng/ml EGF for 15 min. The western blots shown are representative of 3 independent experiments with consistent results.

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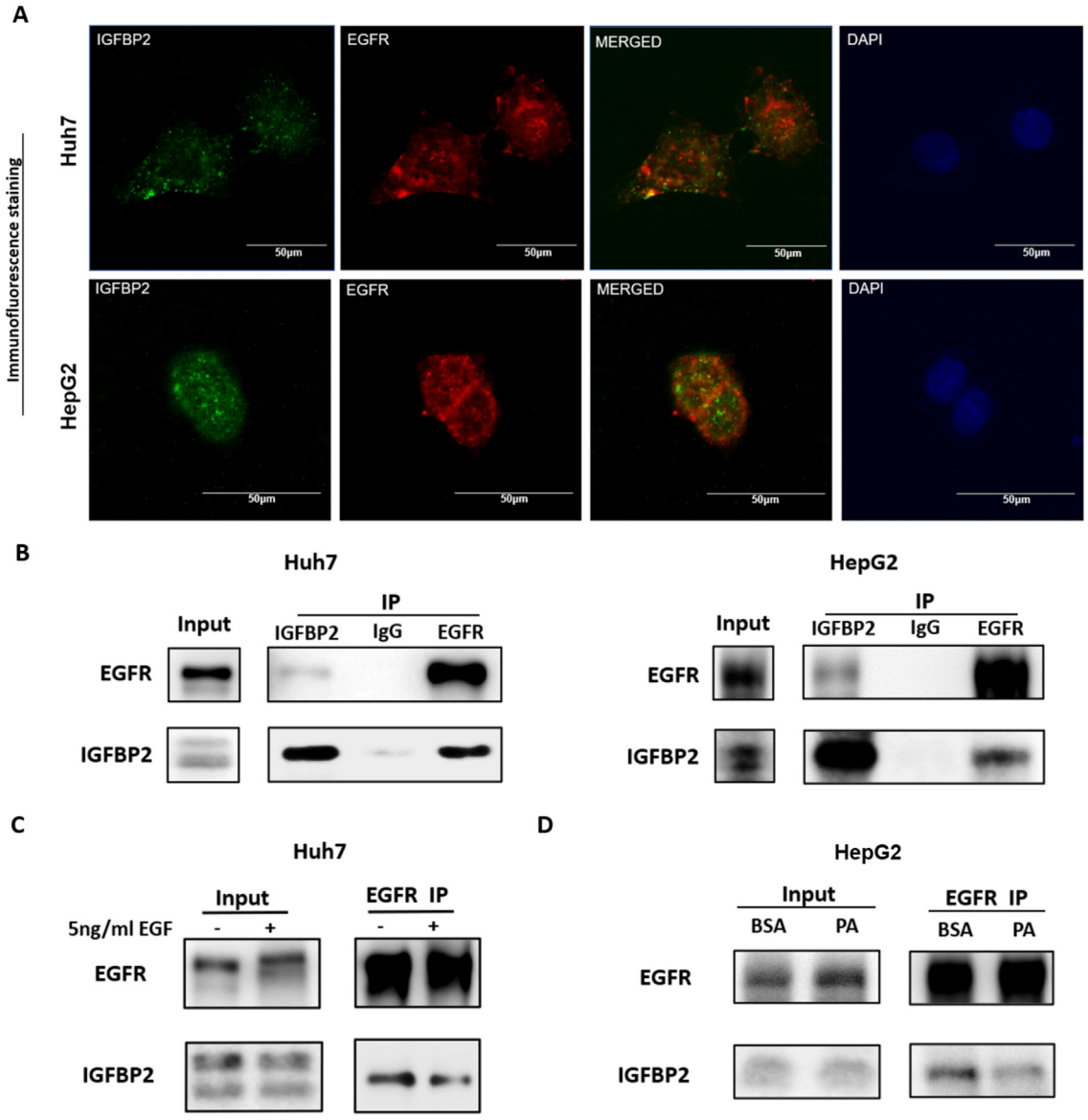

Figure 5: IGFBP2 co-localizes and interacts with EGFR in hepatocytes. (A) Representative images of immunofluorescence staining for IGFBP2 (green), EGFR (red) and DAPI (blue) in HepG2 and Huh7 cells. (B) Co-immunoprecipitation (Co-IP) of endogenous IGFBP2 and EGFR proteins in HepG2 and Huh7 cells analyzed by immunoblotting. (C) Co-IP analysis of the IGFBP2-EGFR complexes in Huh7 cells stimulated with 5 ng/ml EGF for 15 min and in (D) HepG2 cells exposed to 300 μM PA or 1% BSA for 24 h. The IF and western blots shown are representative of 3 independent experiments with consistent results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PA-induced lipid accumulation and lipogenic proteins in both HepG2 and Huh7 cells (Supplementary Figure 11A-D, 14A—B). Moreover, these effects were almost completely reversed by either erlotinib or a specific pharmaceutic inhibitor of STAT3, stattic (Supplementary Figure 11A-D, 14A—B). Likewise, the siRNA-mediated down-regulation of EGFR alleviated PA-induced lipid accumulation (Supplementary Figure 12A—D) and suppressed lipogenic gene expression (Supplementary Figure 12E, F) in both HepG2 and Huh7 cells. By contrast, overexpression of EGFR promoted intracellular TG accumulation and enhanced expression of the genes required for lipogenesis and fatty acid transport. In addition, treatment with erlotinib completely prevented the pro-steatotic effect of EGFR (Supplementary Figure 12G, H). Besides, the specific siRNA targeting on STAT3 had similar effects with EGFR-siRNA on lipid accumulation in HepG2 and Huh7 cells (Supplementary Figure 13A—F). Collectively, these data illustrated a pro-steatotic effect of the EGFR-STAT3 pathway in hepatocytes.

3.6. IGFBP2 knockdown aggravates hepatosteatosis through activating the EGFR-STAT3 pathway

To further ascertain whether the EGFR-STAT3 axis mediates the role of IGFBP2 in hepatic steatosis, we treated with erlotinib or stattic in IGFBP2-deficient hepatocytes. As shown in Figure 6A—B and Supplementary Figure 14C—H, the enhanced lipid accumulation induced by IGFBP2 deficiency was abrogated by erlotinib and stattic treatment. Correspondingly, erlotinib and stattic significantly inhibited the elevated expression of SREBP1 and its target genes in IGFBP2-deficient hepatocytes (Figure 6A, Supplementary Figure 14I—J). These data suggested that the EGFR-STAT3

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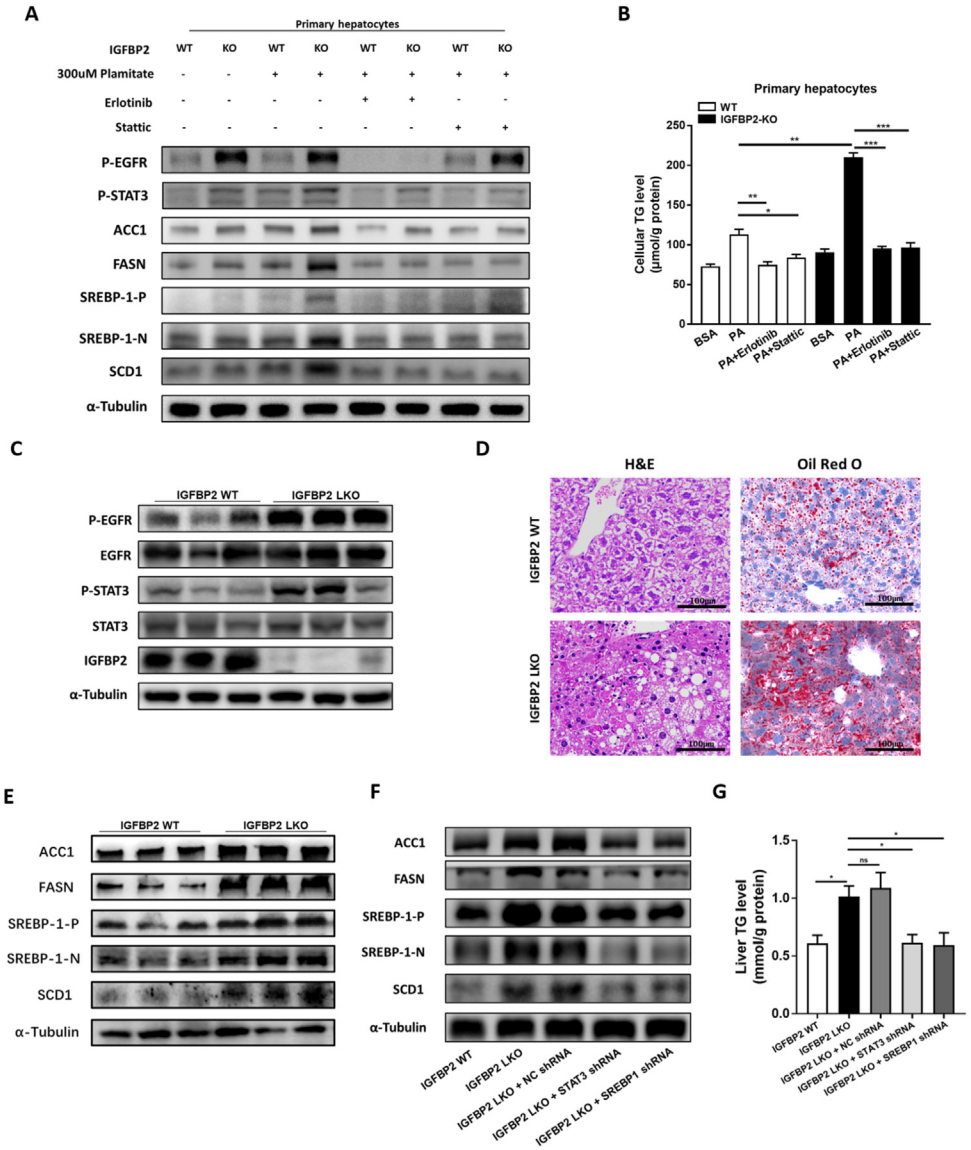

Figure 6: IGFBP2 deletion induces hepatic steatosis through activating EGFR-STAT3 pathway. (A—B) Primary hepatocytes isolated from WT and *Igfbp2*-KO which were treated with 300 μM palmitic acid (PA) or 1% bovine serum albumin (BSA), together with 5 ng/ml EGF, 2 μM erlotinib or 5 μM stattic for 24 h. (A) Western blotting results of protein levels of genes involved in *de novo* lipogenesis in primary hepatocytes. (B) Cellular TG content determination in primary hepatocytes were repeated three times. (C—G) *Igfbp2*-floxed mice were intravenously injected with AAV8-TBG-null (IGFBP2 WT) or AAV8-TBG-Cre (IGFBP2 LKO), and then administered adenoviruses carrying shRNA targeting STAT3 or SREBP1 shRNA (IGFBP2 LKO + STAT3 or SREBP1 shRNA) (n = 6/group). (C) Western blotting results of protein levels of EGFR-STAT3 pathway in the livers of IGFBP2 WT and IGFBP2 LKO mice. (D) Representative images of Oil Red O and H&E staining of liver sections from IGFBP2 WT and IGFBP2 LKO mice. Scale bar, 100 μm. (E—F) Western blotting results of protein levels of genes involved in *de novo* lipogenesis in the livers of mice in each group. (G) The hepatic TG content of mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The western blots shown are representative of 3 independent experiments with consistent results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pathway accounts for, at least partially, the regulatory effect of IGFBP2 on hepatic steatosis. Because STAT3 is a master transcription factor, we examined whether STAT3 directly affects expression of SREBP1 at a transcriptional level. *In silico* prediction revealed two potential bind sites within −2000bp region of *Srebf1* promoter (Supplementary Figure 15A). Indeed, STAT3 significantly enhanced the WT promoter activity of *Srebf1*, as determined by dual-luciferase reporter assays (Supplementary Figure 15B). By contrast, STAT3 had no effect on the mutant of *Srebf1* promoter where the two aforementioned STAT3 binding sites were mutated (Supplementary Figure 15B), indicating that STAT3 has a direct effect on SREBP1 transcription and lipogenic gene regulation. In order to explore whether STAT3 or SREBP1 mediated the regulatory effect of IGFBP2 on NAFLD, the shRNA knockdown adenoviruses which target STAT3 and SREBP1 were administered in IGFBP2 LKO mice. As shown in Figure 6C—G, IGFBP2 LKO significantly promoted EGFR-STAT3 pathway and the expression of SREBP1, SCD1 in mice. Meanwhile, depletion of STAT3 or SREBP1 could fully reversed the NAFLD phenotype caused by IGFBP2 deficiency.

To further assess the role of IGFBP2-EGFR-STAT3-SREBP1 axis in NAFLD, we evaluated their expression in human liver samples by IHC staining. Compared with normal controls, expression levels of SREBP1, phospho-EGFR and STAT3 were substantially increased in the livers of NAFLD patients (Supplementary Figure 16).

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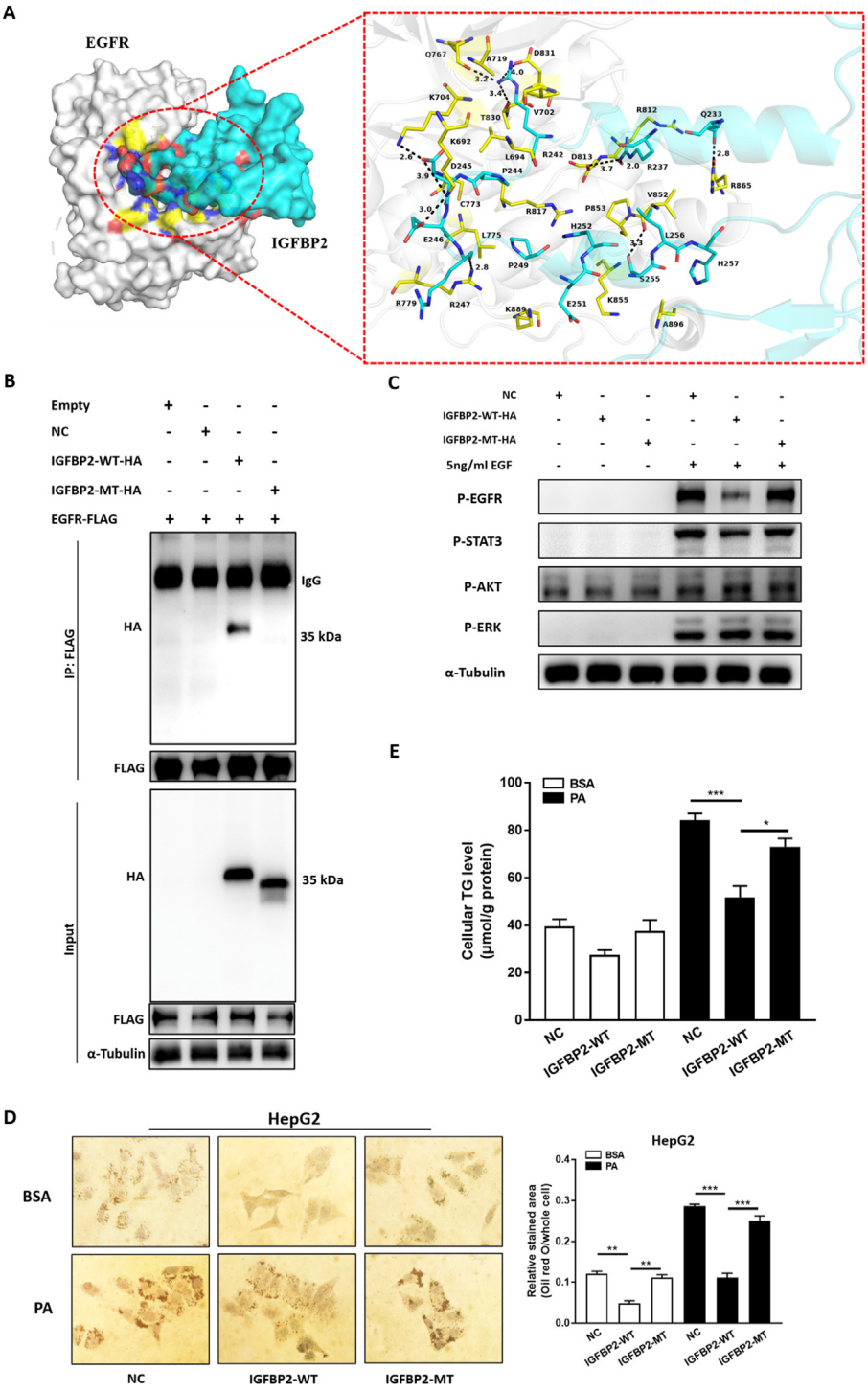

Figure 7: A motif of 233–257 amino acids within IGFBP2 is responsible for its interaction with EGFR. (A) IGFBP2-EGFR docking model visualized by PyMol. The surface of EGFR protein is marked with white, and the surface of IGFBP2 protein is marked with cyan. The amino acids interacting with IGFBP2 in EGFR protein are represented by yellow stick, and the amino acids interacting with EGFR in IGFBP2 protein are represented by cyan stick. At the end of the stick, blue represents nitrogen atoms and red represents oxygen atoms. (B) Co-IP of IGFBP2-EGFR complexes was performed in HEK-293T cells that were transfected with a lentiviral plasmid containing Flag-tagged EGFR and then co-transfected with blank, IGFBP2-WT-HA, IGFBP2-MT-HA or an empty control (NC) plasmids. (C–E) HepG2 cells were transfected with blank, NC, IGFBP2-WT-HA or IGFBP2-MT-HA plasmids, and then treated with 5 ng/ml EGF for 15 min, 300 μM PA or 1% BSA for 24 h. (C) Immunoblotting analysis of the EGFR signaling pathway. (D) Representative Oil Red O staining of three independent experiments (left), quantification of Oil Red O staining positive area (Right). (E) Cellular TG content determination was repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The IP and western blots shown are representative of 3 independent experiments with consistent results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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3.7. IGFBP2 233—257 amino acids play a key role in the EGFR-IGFBP2 interaction

Given the direct interaction of IGFBP2 with EGFR existed in hepatocytes and played an important role in steatosis, we wanted to figure out a specific structural basis for IGFBP2 binding to EGFR. To this end, we firstly performed molecular docking calculation by using protein structural models of EGFR and IGFBP2 through bioinformatics analysis. As visualized by PyMol, a predicted stabilized complex of EGFR-IGFBP2 was generated, and the motif responsible for IGFBP2 binding to EGFR were mainly concentrated at 233—257 in its sequence, i.e., Glb233, Arg237, Arg242, Pro244, Asp245, Glu246, Arg247, Pro249, Glu251, His252, Ser255, Leu256, His257 (Figure 7A). Accordingly, we generated two transfection plasmids that contains either a full-length (IGFBP2-WT) or truncated mutant (233—257 aa deleted) of IGFBP2 and then examined their ability to interact with EGFR. In line with the endogenous IGFBP2-EGFR interaction as documented above in Figure 5, the interaction occurred between these two enforced over-expressed proteins in HEK293T cells co-transfeted with IGFBP2-WT and EGFR (Figure 7B). However, the IGFBP2 mutant was unable to interact EGFR (Figure 7B), indicating that the 233—257 aa motif is crucial for IGFBP2 binding to EGFR.

Next, we investigated whether 233—257 aa in IGFBP2 was essential for its effect on the EGFR-STAT3 signaling and steatosis in hepatocytes. As shown in Figure 7C, overexpression of IGFBP2-WT suppressed the EGF-induced EGFR and STAT3 activation in HepG2 cells, without obvious effect on the Akt and ERK pathways, which was consistent with the findings steamed from the IGFBP2-deficient hepatocytes. Of note, none of these arms of the EGFR signaling was influenced in the cells overexpressing the IGFBP2 mutant lacking 233—257 aa (Figure 7C). Moreover, compared with the controls, overexpression of IGFBP2-WT significantly alleviated lipid accumulation and decreased intracellular TG levels in HepG2 cells. However, the IGFBP2 mutant had no effect on hepatic steatosis (Figure 7D,E), suggesting the motif of 233—257 aa in IGFBP2 is essential for its interaction with EGFR and accounts for the effect on steatosis in hepatocytes. Further, the AAV8-TBG-*Igfbp2*-WT, AAV8-TBG-*Igfbp2*-MT (213—237 aa deleted) or AAV8-TBG-GFP vector were constructed and administered in *ob/ob* mice. As shown in Figure 8, IGFBP2 liver specific overexpression significantly alleviated the hepatic steatosis, and suppressed the EGFR-STAT3 pathway and the expression of SREBP1, SCD1 in *ob/ob* mice. Meanwhile, depletion of 213—237 aa fully abrogated the effect of IGFBP2 on EGFR-STAT3 pathway and hepatic steatosis.

4. DISCUSSION

In the present study, we have provided both experimental evidence and mechanistic data showing that IGFBP2 functions as an endogenous protector against hepatosteatosis through inhibiting the EGFR-STAT3 signaling pathway. First, hepatic expression of IGFBP2 had a robust decrease in all the 8 available human datasets that we analyzed regardless the differences in age, gender and region throughout the whole subjects. The decreased expression of IGFBP2 mRNA and protein levels were further confirmed in our NAFLD patients and obese animal models. Second, *Igfbp2* deletion markedly aggravated the development and progression of NAFLD and NASH as reflected in more serious, hepatic steatosis, inflammation, fibrosis, apoptosis and insulin resistance in DIO mice. Third, IGFBP2-deficient hepatocytes significantly heightened lipogenic gene expression and lipid accumulation. Forth, IGFBP2 directly binds to and functionally interacts with EGFR. Blockage of the interaction by knockdown of IGFBP2 resulted in activation of EGFR and the downstream STAT3 pathway, enhancing the

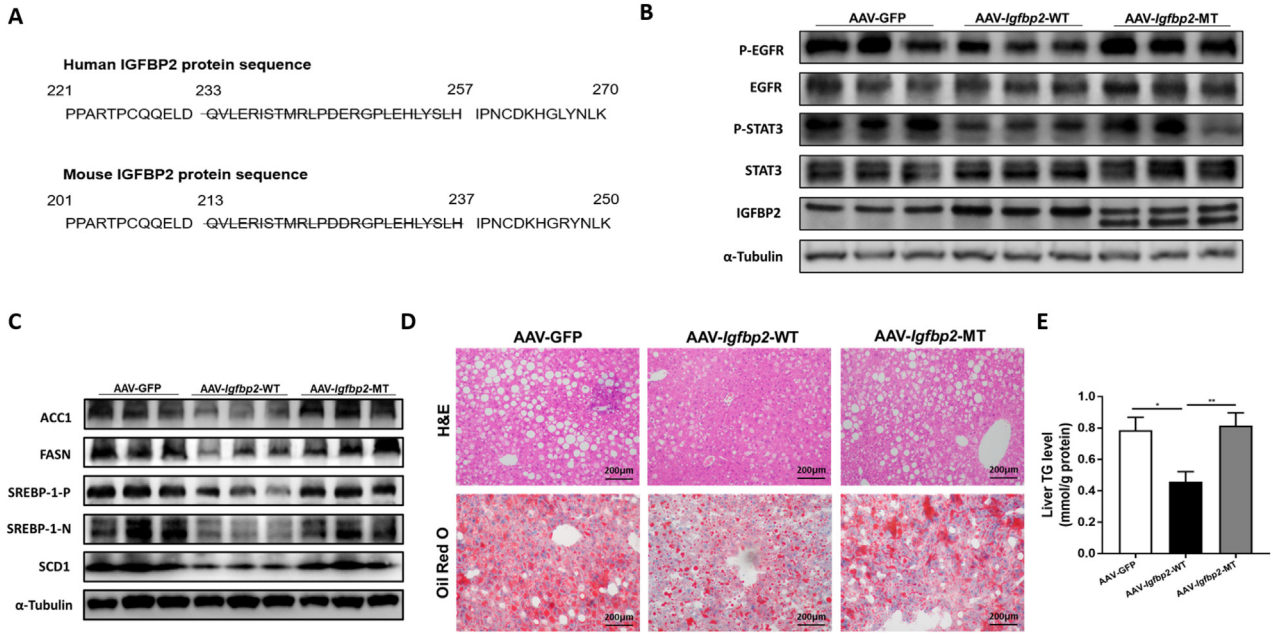

Figure 8: IGFBP2 suppresses EGFR-STAT3 pathway and prevents hepatic steatosis through the 213—237 aa in mice. (A—D) Ob/ob mice were administered with AAV8-TBG-*Igfbp2*-WT, AAV8-TBG-*Igfbp2*-MT (213—237 aa deleted) or AAV8-TBG-GFP (n = 6/group). (A) Schematic of the truncated mutant (233—257 or 213—237 aa were deleted) of human or mouse protein sequence of IGFBP2. (B) Western blotting results of hepatic protein levels of EGFR-STAT3 pathway of mice in each group. (C) Western blotting results of hepatic protein levels of genes involved in *de novo* lipogenesis of mice in each group. (D) Representative images of Oil Red O and H&E staining of liver sections from mice in each group. Scale bar, 200 μm. (E) The hepatic TG content of mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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promoter activity of *srebf1* that governs the transcription of multiple key lipogenic genes. Furthermore, the 233—257 aa motif in IGFBP2 was identified to be essential for its binding to EGFR and the consequent functions on the regulation of EGFR-STAT3 signaling and hepatic steatosis. Overall, our study has uncovered IGFBP2 as a novel regulator of the EGFR-STAT3 signaling and a key protector against hepatic steatosis. Thus, the IGFBP2-EGFR-STAT3 axis may serve as a promising intervention target for prevention and treatment of NAFLD and NASH (Figure 9).

Nowadays, microarrays and sequencing technologies have been widely used over the biomedical research fields, including NAFLD researches in either patients or experimental animals, while many variables in, such as, analysis platforms, sample sizes and sources often bring in inconsistent outcomes. RRA algorithm has strong robustness to noise and was therefore adopted in the current study for the integrated analysis. We found that hepatic expression of IGFBP2 was consistently down-regulated in NAFLD subjects within all datasets that we analyzed, which agrees with a lower level of circulating IGFBP2 detected in NAFLD patients [29]. Moreover, the relatively lower level of circulating IGFBP2 was also found in the subjects with obesity [30], T2DM [18] and metabolic syndrome (MetS) [31]. The circulating levels of IGFBP2 are inversely associated with several metabolic indices, including body mass index (BMI), plasma glucose and insulin, triglycerides and ALT activity [18,30,31], suggesting a beneficial effect of IGFBP2 on the regulation of metabolic homeostasis. It has been reported that *Igfbp2* gene expression is potentially regulated via its promoter hypermethylation and that early hypermethylation of hepatic *Igfbp2* results in its reduced expression and precedes fatty liver in mice [32,33]. By contrast, overexpression of IGFBP2 has been shown to improve insulin sensitivity and reverse diabetes in DIO, *ob/ob* or streptozotocin-induced diabetic mice [34], further supporting an important regulatory role of IGFBP2 in metabolism.

Notably, previous studies have shown that mice with targeted ablation of IGFBP2 were not accompanied by major alteration in metabolic phenotypes under the normal basal conditions [35,36]. However, in the presence of nutritional energy excess, *Igfbp2*-deficiency profoundly proceeded the development and progression of NAFLD and NASH in either 8- or 20-weeks DIO mouse models. Hepatic steatosis is chiefly attributed to the excessive lipid droplets that are often derived from increased FFAs and lipid synthesis owing to the over-nutrition. The main transcriptional factors of lipid synthesis include SREBP1, liver X receptor (LXR), carbohydrate-responsive element-binding proteins (ChREBP) and hepatocyte nuclear factor 4 alpha (HNF4α). Our findings indicated that IGFBP2 deletion aggravated hepatic lipid accumulation by driving the activation of SREBP1 downstream gene network, accompanying with more serious liver damage and dyslipidemia. Because NAFLD is intensively related to insulin resistance, it is not surprising that *Igfbp2*-KO mice exhibited more sensitive to diet-

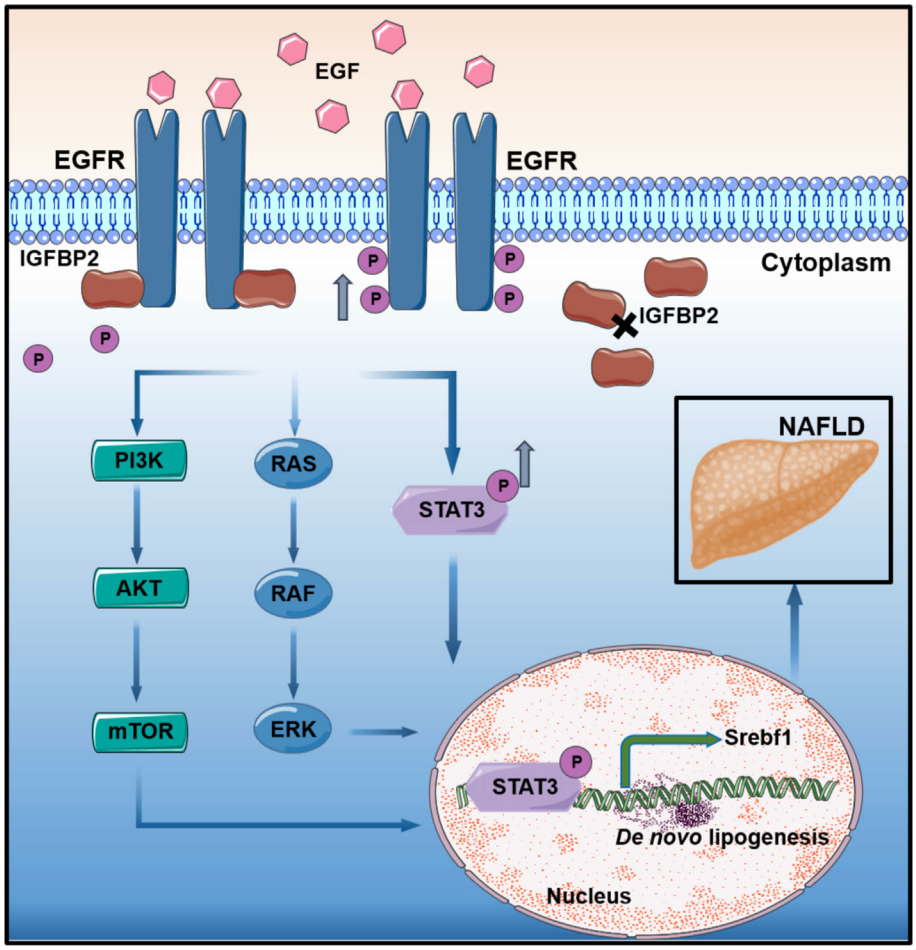

Figure 9: A schematic model depicting that IGFBP2 specifically binds to and interacts with EGFR, resulting in suppression of the STAT3 pathway-mediated progression of hepatic steatosis.

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induced obesity and more severe insulin resistance, compared with WT DIO mice. It is noted that IGFBP2 was previously reported to directly interfere adipogenesis in the differentiated 3T3-L1 cells [37], which may partially account for the enhanced obese phenotype in *Igfbp2*-KO mice. It is well-established that obesity is a significant contributor to the development of fatty liver disease. To definitively ascertain whether the protective role of IGFBP2 against fatty liver is independent of its effects on obesity, we conducted IGFBP2 liver-specific loss- and gain-of-function experiments. These findings showed that the protective effects of IGFBP2 against NAFLD persisted, which could further rule out the effect of obesity or IGFBP2 produced by other tissues on hepatic steatosis. In addition, we observed hyperglycemia, hyperinsulinemia and impaired IPGTT/IPITT in *Igfbp2*-KO mice, indicating a state of glucose dysregulation and insulin resistance. Consistently, previous study indicated that overexpression of IGFBP2 could improve hepatic insulin resistance and diabetic phenotype via suppressing hepatic glucose production [34].

IGFBP2 was originally characterized as a binding protein and modulator of IGF-1 and -2 [22,23]. Indeed, by modulating the activity of IGF-1, IGFBP2 was shown to reduce postnatal body weight gain in the transgenic mice [37,38]. Besides, emerging evidence has supported the IGF-independent actions of IGFBP2 mediated by its various functional motifs, including nuclear localization signal (NLS, 179—184 aa), heparin-binding domain (HBD, 227—244 aa) and RGD integrin-recognition motif (265—267 aa) [39]. Reyer et al. reported that the RGD motif of IGFBP2 is required for the impaired glucose clearance and intracellular translocation of glucose transporter type 4 (GLUT4) in *Igfbp2*-transgenic mice [40]. Another study reported that HBD synthetic peptides have a comparable effect with native IGFBP2 on the suppression of adipogenesis [41]. Recently, an increasing number of studies have suggested that IGFBP2 functions as a hepatokine or cytoplasmic signaling regulator in an IGF-independent manner, especially in tumor cells. For example, through its RGD sequence, IGFBP2 interacts with integrins and results in activation of the nuclear factor-κB (NF-κB), ERK and PI3K/AKT signaling pathways in several experimental models [42—44]. In addition, owing to the presence of a classic NLS, IGFBP2 is able to enter nuclear affecting the promoter activity of VEGF [45]. In glioma cells, IGFBP2 was first reported by Chua et al. to bind to EGFR with its NLS sequence and potentiates nuclear EGFR accumulation and activation (25). We reported herein that IGFBP2 was able to directly bind to EGFR and regulate the activation of EGFR-STAT3 in hepatocytes, whereas we did not detect any alterations in nuclear translocation of EGFR in the presence or absence of EGF stimulation. The discrepancy between Chua's and our findings is likely attributable to the difference in cell types and experimental systems applied in the two studies. The major findings in Chua's study were principally steamed from a glioma cell line with enforced overexpression of IGFBP2 (25), whereas we found the physical protein-protein interaction between the two endogenous proteins, which is more physiologically relevant. Interestingly, the complex of IGFBP2-EGFR appears critical for maintaining an inactive status of STAT3 in hepatocytes, as IGFBP2 knockdown specifically augmented the EGFR-STAT3 pathway activation. Notably, the increased activity of EGFR and STAT3 was previously observed in DIO mice [14,46]. It is well-established that EGFR is a transmembrane protein with ligand-induced tyrosine kinase activity and plays an important role in multiple biological processes, including metabolism regulation [47]. EGFR TKI treatment has been previously reported to ameliorate hepatic steatosis, fibrotic phenotype and liver injury [12,13,15]. More concretely, EGFR TKI markedly reversed fast-food diet (FFD)-induced transcription of lipogenic genes, including SREBP1, ChREBP, FASN, SCD1 and ACC1 as well as the lipid droplet-related genes, including perilipins (PLINs), G0/G1 switch-2 (G0S2), cell death—inducing DFFA-like effector A (CIDEA) [13]. In line with these findings, *Igfbp2*-deficiency resulted in a significant increase in the EGFR-STAT3 pathway, leading to enhanced expression of the gene network associated with lipogenesis and steatosis in hepatocytes.

Previous studies showed that EGFR can directly interact with and phosphorylate STAT3 at Tyr-705 and thereby activates it for DNA binding upon EGF stimulation [48]. Specific tyrosine residues in the cytoplasmic terminus of the EGFR have been identified to be essential for STAT3 activation. Several tyrosine residues in EGFR C-tail, including Tyr^{992}, Tyr^{1068}, Tyr^{1086}, Tyr^{1148} and Tyr^{1173}, have been previously deemed as autophosphorylation sites. Consistently, STAT3 was proven to preferentially interacts with Tyr^{1068} in the Y*XX*Q sequence that is a STAT3-preferential binding motif [49]. In line with this, we found that phosphorylation of Tyr^{1068} within EGFR intracellular domain was dramatically elevated following EGF stimulation in IGFBP2-deficient hepatocytes. Of note, for the first time, we identified that IGFBP2 occupies the autophosphorylation sites within EGFR C-tail through the sequence of 233—257 amino acids, which prevents EGFR phosphorylation and STAT3 activation, providing an insight into a mechanistic regulation of the EGFR-STAT3 pathway by IGFBP2.

STAT3 has been regarded as a master transcription factor participating in gene regulation related to lipid and glucose metabolism, whereas its physiological or pathological role in the liver remains controversial. It has been reported that IL-6 and IL-22 were able to inhibit lipogenesis via STAT3 activation in the liver, suggesting an effect of anti-steatosis [50,51]. By contrast, it was found that plasma levels of TG and TC were elevated in hepatic STAT3-overexpressing mice, likely through up-regulation of FASN, ACC1 and suppression of acyl-CoA oxidase expression in hepatocytes [52]. Furthermore, previous studies have shown that the STAT3 signaling pathway mediated lipid synthesis by regulating SREBP1 in DIO or diabetic mice [53]. In agreement with the pro-steatotic effect of STAT3 as reported previously, we found that inhibition of STAT3 by using pharmaceutic or genetic means efficiently reversed the lipid accumulation induced by EGF or knockdown of IGFBP2 in hepatocytes. Furthermore, overexpression of STAT3 significantly enhanced the WT promoter activity of *Srebf1*, but not the mutant that lacks of STAT3 binding sites, suggesting a direct effect of STAT3 in the promoter activity of *Srebf1*.

In conclusion, our findings have uncovered a novel role of IGFBP2 as an endogenous protector against hepatic steatosis through the suppression of the EGFR-STAT3 axis by the IGFBP2-EGFR complex. In this regard, the clinical efficacy of the well-designed EGFR inhibitors for tumor therapeutics may open a potential possibility for its application in the treatment of NAFLD/NASH and the associated diseases. However, strictly different from that used in cancer, complete blocking of EGFR in NAFLD/NASH may result in unexpected side effects, such as imbalance of cell survival and proliferation [47,54]. Pharmaceutically targeting the IGFBP2-EGFR-STATs axis, rather than EGFR itself, may be a more suitable way to the treatment of NAFLD/NASH and related disorders, which deserves further investigations.

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ETHICAL STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of Zhongshan Hospital of Fudan University. The patients/participants provided their written informed consent to participate in this study.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Tianyu Zhai: Writing — review & editing, Writing — original draft, Funding acquisition, Data curation, Conceptualization. **Liang Cai:** Project administration. **Xi Jia:** Project administration. **Mingfeng Xia:** Methodology. **Hua Bian:** Methodology. **Xin Gao:** Resources, Methodology. **Chenling Pan:** Methodology. **Xiaoying Li:** Supervision. **Pu Xia:** Writing — review & editing, Writing — original draft, Methodology, Funding acquisition, Formal analysis, Conceptualization.

ACKNOWLEDGEMENTS

The bioinformatics analysis was supported by the Medical Science Data Center of Fudan University. The schematic diagram of the research mechanism was drawn by Figdraw.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest that pertain to this work.

DATA AVAILABILITY

Data will be made available on request.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2024.102026.

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