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DDB1 E3 ligase controls dietary fructose-induced ChREBPα stabilization and liver steatosis via CRY1

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Abstract

Fructose over-consumption contributes to the development of liver steatosis in part by stimulating ChREBPα-driven de novo lipogenesis. However, the mechanisms by which fructose activates ChREBP pathway remain largely undefined. Here we performed affinity purification of ChREBPα followed by mass spectrometry and identified DDB1 as a novel interaction protein of ChREBPα in the presence of fructose. Depletion and overexpression of *Ddb1* showed opposite effects on the ChREBPα stability in hepatocytes. We next tested the impact of hepatic *Ddb1* deficiency on the fructose-induced ChREBP pathway. After 3-week high-fructose diet feeding, both *Ddb1 liver-specific knockout* and AAV-TBG-Cre-injected $Ddb1^{flox/flox}$ mice showed significantly reduced ChREBPα, lipogenic enzymes, as well as triglycerides in the liver. Mechanistically, DDB1 stabilizes ChREBPα through CRY1, a known ubiquitination target of DDB1 E3 ligase. Finally, overexpression of a degradation-resistant CRY1 mutant (CRY1–585KA) reduces ChREBPα and its target genes in the mouse liver following high-fructose diet feeding. Our data revealed DDB1 as an intracellular sensor of fructose intake to promote hepatic de novo lipogenesis and liver steatosis by stabilizing ChREBPα in a CRY1-dependent manner.

Keywords

DDB1; ChREBPα; CRY1; Fructose; De novo lipogenesis; Liver steatosis

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CRediT authorship contribution statement

Xin Tong: Conceptualization, Methodology, Writing - original draft, Formal analysis, Investigation, Visualization. **Deqiang Zhang:** Investigation, Visualization. **Omar Shabandri:** Investigation. **Ethan Jin:** Investigation. **Kenneth Stamper:** Investigation. **Meichan Yang:** Investigation. **Zifeng Zhao:** Investigation. **Lei Yin:** Conceptualization, Methodology, Writing - original draft, Formal analysis.

Declaration of competing interest

No conflicts of interests relevant to this work were reported.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2020.154222.

1. Introduction

Dietary components have significant impact on liver lipid metabolism. In particular, dietary fructose activates glycolysis and de novo lipogenesis, leading to triglyceride accumulation in hepatocytes [1,2]. Chronic feeding of mice with high-fructose diet results in weight gain, liver steatosis, and subsequently metabolic impairment [1,3]. Previous reports, including ours, demonstrated a potent effect of fructose on de novo lipogenesis in hepatocytes [4,5]. Fructose metabolism not only provides substrates for de novo lipogenesis but also activates the transcription of lipogenic enzymes mainly via the lipogenic factor ChREBP [5,6]. We reported that short-term high-fructose diet induces the expression of lipogenic enzymes and simple steatosis in WT mice but not *Chrebp*$^{-/-}$ mice [4]. Such an induction of the ChREBP pathway could be an adaptive response to store excess energy in the liver in response to fructose intake. So far, how fructose activates hepatic ChREBP pathway is largely unclear.

ChREBPα plays a major role in driving the transcription of lipogenic enzymes in hepatocytes [7–9]. Its targets include *Fasn* (fatty acid synthase), *Acc1* (acetyl-CoA carboxylase), *Scd1* (stearoyl-CoA desaturase 1), *Lpk* (liver-pyruvate kinase), and *Gck* (glucokinase) [6]. A panel of studies has elucidated how glucose regulates ChREBPα via post-translational modifications to affect ChREBPα nuclear localization, transcriptional activity, and protein-protein interactions [9]. In particularly, in the presence of high glucose, ChREBPα is modified by both acetylation and O-GlcNAcylation. Glucose-induced acetylation of ChREBPα on lysine 672 depends on the activity of the histone acetyl-transferase coactivator p300 [10]. *O*-GlcNAc transferase (OGT) has been shown to mediate the GlcNAcylation of ChREBPα [10]. Interestingly, both acetylation and *O*-GlcNAcylation levels of ChREBPα were shown to be elevated in the liver of *ob/ob* mice, contributing to the enhanced ChREBPα activity and fat accumulation [10,11]. In contrast, how fructose impacts post-translational modifications of ChREBPα remains largely unexplored.

Recently, Herman et al. discovered a novel isoform of ChREBP, namely ChREBPβ, in adipose tissue to induce de novo lipogenesis [12]. Compared with ChREBPα, ChREBPβ lacks both the nuclear export sequence and the glucose-sensing domain. As a result, ChREBPβ was assumed to be constitutively active regardless of intracellular glucose concentration. Subsequently, the Chrebpβ mRNA was also detected in other metabolic tissues, including liver and pancreatic islets [13]. However, whether both ChREBP isoforms are similarly regulated by fructose has not been examined yet.

DDB1 (DNA-damaging binding protein) is a scaffolding protein in the CUL4A-DDB1 E3 ligase complex [14], which is known for promoting the ubiquitination and degradation of a number of substrates including p27, CDT1, and p53 [15–17]. We first reported that DDB1 is important for the mammalian molecular circadian clock and glucose metabolism in the liver [18,19]. Specifically, hepatic DDB1 promotes the FOXO1-driven gluconeogenesis by degrading the clock protein CRY1, which interacts with the nuclear FOXO1 protein [18]. Given the elevated level of DDB1 in the liver of high-fructose diet-fed mice, we speculated that DDB1 might regulate lipid metabolism as well.

In the current study, we identified DDB1 as a novel binding protein for both ChREBPα and ChREBPβ hepatocytes treated with fructose. We demonstrated that DDB1 protects the ChREBPα stability via the DDB1 target, CRY1, in hepatocytes. Chronic or acute deletion of *Ddb1* in hepatocytes results in markedly reduced levels of ChREBPα, lipogenic genes, and lowered triglycerides (TG) in the liver of high-fructose diet-fed mice. In summary, we uncovered a novel molecular pathway that links ChREBPα-mediated-de novo lipogenesis and the DDB1-driven CRY1 degradation pathway in response to dietary fructose.

2. Research methods and materials

2.1. Animals and treatments

Animal experiments were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee of University of Michigan Medical School. *Ddb1*$^{flox/flox}$ mice were backcrossed to the *C57BL/6J* background more than nine generations. Liver-specific *Ddb1* knockout *(Ddb1-LKO)* mice were generated by crossing *Ddb1*$^{flox/flox}$ mice with *Albumin-Cre* mice purchased from the Jackson Laboratory. All mice were housed on a 12 h:12 h light/dark cycle at 25 °C with free access to water and regular chow (26.8% kcal from protein, 16.6% from fat, and 56.4% from starch) or high-fructose diet (Research Diets; 20% kcal from protein, 10% kcal from fat, and 70% from free fructose) for 3 weeks. In a separate experiment, one week after injection with AAV-TBG-GFP or AAV-TBG-Cre, 8-week old *Ddb1*$^{flox/flox}$ mice were fed high-fructose diet for 3 weeks. In the third experiment, 8 to 10-week old WT mice were fed high-fructose diet for 10 days and injected with Ad-GFP or Ad-CRY1585KA before high-fructose diet for 1 week.

2.2. Primary mouse hepatocyte isolation and culture

Primary mouse hepatocytes were isolated from C57BL/6 male mice with PCR-confirmed genotypes (9–10 weeks). The liver was perfused with 15 mL of EBSS (Invitrogen) with 0.5 mM EGTA for 5 min, followed by perfusion with 15 mL of 100 U/mL type I collagenase (Worthington) dissolved in HBSS (Invitrogen, containing 10 mM HEPES, 4 mM NaOH, and 10 mM $CaCl_2$) via the inferior vena cava for 5 min. After dissection, hepatocytes were released by scattering with tweezers, passed through a 100-μm cell strainer, and then spun at 50 ×*g* for 1 min. The pellet was re-suspended in DMEM and then spun at 50 ×*g* for 10 min in a Percoll gradient to remove dead hepatocytes. Viable cells were washed with DMEM at 50 × g for 10 min and checked by trypan blue staining. Primary mouse hepatocytes in DMEM with 5% FBS were seeded at a density of 2×10^5 cells/well in 12-well-plates. Cells were transduced with adenoviruses within 6 h post seeding and treated with fructose 24 h post seeding.

2.3. Proteomic analysis

About 1×10^8 293A cells were transduced with Ad-GFP or Ad-FLAG-Chrebpα and then exposed to 25 mM fructose for 16 h before immunoprecipitation with anti-FLAG M2 agarose beads overnight. The immunocomplex was washed with IP buffer (150 mM NaCl, 50 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% NP-40) 5 times and then eluted with 1× SDS buffer. To separate and visualize the ChREBPα-containing complex, both Ad-GFP and Ad-FLAG-ChREBPα pull-down samples were loaded onto 9% SDS-PAGE gel and then stained with

Colloidal Blue solution (Invitrogen). Four distinctive bands from Ad-Flag-ChREBPα and the corresponding gel from Ad-GFP lanes were cut out and submitted to the Proteomics Resource Facility in the Department of Pathology of University of Michigan Medical School for mass spectrometry.

2.4. Protein extraction, immunoprecipitation, and ubiquitination assay

Liver tissues or cell pellets were lysed in hypotonic buffer (5 mM Tris-HCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 8% Sucrose). After centrifuge, pellets were washed once with hypotonic buffer and re-suspended in RIPA buffer (5 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.05% deoxycholic acid, 10% Glycerol) prior to sonication for 5 s. Nuclear fractions were then collected after centrifugation at 13,000 rpm × 10 min. FLAG-M2 beads or streptavidin beads were added into nuclear fractions to capture FLAG-CRY1 or CBP-CRY1. The protocol for detecting ubiquitination was reported [20] with minor modifications. Anti-ChREBP was used to pull down poly-ubiquitinated ChREBPα conjugates. Western blot analysis was performed using the following primary antibodies: anti-DDB1 (Abcam ab9194), anti-CRY1 (sc-101006), anti-GAPDH (sc-25778), anti-Lamin A/C (sc-20681), anti-CBP (sc-33000) (Santa Cruz biotechnology), anti-ChREBP (Novus NB400–135), anti-ubiquitin (Sigma U5379), and anti-β-tubulin (T5201) (Sigma-Aldrich).

2.5. Generation and injection of recombinant adenoviruses

Adenoviruses including Ad-shLacZ, Ad-shDdb1, Ad-Cry1-WT-Flag, and Ad-Cry1–585KA-Flag, Ad-Flag-ChREBP and Ad-Flag-Ddb1 were described [19]. Ad-Flag-Chrebpβ virus was generated after deleting the N terminal 77-aa-coding sequence of Chrebpα via QuikChange PCR. AAV-TBG-CRE and AAV-TBG-GFP were purchased from the UPENN Vector Core. For adenoviral injections, 1×10^{12} pfu per adenovirus were administrated via tail-vein injection. For each virus, a group of 4 to 5 mice was injected with the same dose of viral particles. 10–14 days after injection, liver tissues were harvested at ZT8 for protein and RNA analysis.

2.6. Statistical analysis

All data are reported as mean ± SD. Differences between two groups were assessed by two-tailed Student's *t*-test. Differences between more than two groups were analyzed by ANOVA followed by Tukey's post-hoc testing. p value < 0.05 was deemed as statistically different.

2.7. Data and resource availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

3. Results

3.1. Identification of DDB1 as a fructose-induced interacting protein of ChREBPα

We reported that even 1-week high-fructose diet potently induces both ChREBPα mRNA and protein levels in hepatocytes and liver [4]. We then asked whether high-fructose diet could increase the ChREBPα stability in addition to its enhanced transcription. In a

cycloheximide chase experiment, FLAG-tagged ChREBPα showed a half-life of approximately 2 h in primary mouse hepatocytes from mice on regular chow, whereas the half-life of ChREBPα was extended to about 5 h in those from mice on high-fructose diet (Fig. 1A). Moreover, fructose treatment was sufficient to reduce the polyubiquitination of ChREBPα in those cells (Supplementary Fig. 1A). Thus, fructose increases the ChREBPα stability by suppressing its ubiquitination-dependent degradation.

To further explore how fructose regulates the ChREBPα stability, we employed an unbiased proteomic approach to identify novel ChREBPα-binding protein(s) that could promote the ChREBPα stability. We transduced 293A cells with either Ad-GFP or Ad-Flag-Chrebpα and incubate cells in growth medium containing 25 mM fructose for 16 h before immuno-precipitation with anti-FLAG M2 beads. Four distinct bands in the Ad-Flag-Chrebpα lane on an SDS-PAGE gel were cut out for mass spectrometry analysis (Fig. 1B). One of the identified interaction proteins was DDB1 (Fig. 1C), a key component of DDB1-CUL4A E3 ligase complex known for its function in promoting the ubiquitination and degradation of a number of substrates important for cell cycle regulation [14]. Our lab has previously discovered that DDB1 also promotes hepatic gluconeogenesis via CRY1 degradation [18,19]. To validate such an interaction, we firstly performed co-immunoprecipitation assay in both primary mouse hepatocytes and the mouse liver. In primary mouse hepatocytes transduced with Ad-Flag-ChREBPα and cultured in the presence of fructose, FLAG-ChREBPα was shown to interact strongly with the endogenous DDB1 (Fig. 1D). Furthermore, we detected the interaction of FLAG-ChREBPα with the endogenous DDB1 in the fructose-fed mouse liver (Fig. 1E). We then compared ChREBPα with ChREBPβ and found both isoforms interacted with the endogenous DDB1 (Supplementary Fig. 2A). Therefore, we discovered and confirmed that DDB1 is a novel interaction protein of ChREBPα in both fructose-treated hepatocytes and high-fructose diet-fed mouse liver. Since ChREBPα is highly enriched in the liver, our current study focuses on how DDB1 regulates hepatic ChREBPα stability and activity during fructose intake.

To test whether the protein-protein interaction of ChREBPα with DDB1 specifically occurs in the presence of fructose but no other monosaccharides, we performed co-immunoprecipitation assay with cells cultured in either 25 mM glucose or 25 mM fructose. Although DDB1 was detectable in cells treated with glucose after immunoprecipitation of FLAG-ChREBPα, the amount of DDB1 was about 6-fold higher in the presence of 25 mM fructose (Fig. 1F). These results indicate that fructose potently enhances the formation of ChREBPα-DDB1 complex in hepatocytes. Furthermore, we examined the effect of increasing doses of fructose on the DDB1- ChREBPα interaction in primary mouse hepatocytes. The strong interaction between DDB1 and ChREBPα was detected at 5 mM of fructose treatment (Fig. 1G) even though all the cells were incubated in medium culture with 25 mM glucose. These data suggest the ChREBPα-DDB1 protein complex is robustly formed in response to fructose. We also explored the potential signaling pathways that may promote or suppress the fructose-induced ChREBP-DDB1 interaction. Given the importance of the AKT-mTOR pathway in nutrient sensing and lipid biosynthesis [21], we firstly asked whether inhibition of this pathway had any effects on ChREBPα-DDB1 interaction following fructose stimulation. MK2206 [22] and Rapamycin [23] were used in Huh7 cells to inhibit the AKT and mTORC1 activity respectively. Indeed when AKT and mTOR

pathways were inhibited, the amount of DDB1 associated with ChREBPα was markedly reduced even though both inhibitors had minimal effects on the ChREBPα levels (Supplementary Fig. 3). These results suggest the lipogenic AKT-mTORC1 pathway could be important for the DDB1-ChREBPα complex formation in hepatocytes in response to fructose.

ChREBPα and DDB1 are different proteins with distinctive functional motifs. ChREBPα is a helix-loop-helix (HLH)-containing transcription factor [8], whereas DDB1 is a scaffolding protein that functions to connect CUL4A with substrate-binding proteins [l4]. To map the interaction domains, we generated a series of deletion mutants of both ChREBPα and DDB1 for co-immunoprecipitation assay. Our results showed the C-terminal regions of both proteins are required for the interaction between those two proteins (Supplementary Fig. 4A–B). Of note, both ChREBPα and ChREBPβ share the C-terminal region, which can explain these two isoforms could interact with DDB1.

3.2. DDB1 is both necessary and sufficient to promote the ChREBPα stability and activity in hepatocytes

DDB1 is known for its function in promoting substrate ubiquitination and degradation. To assess the impact of *Ddb1* depletion on the ChREBPα stability and ubiquitination, we first harvested primary mouse hepatocytes from Ad-shDdb1-injected mice after 3 days of high-fructose diet feeding. Acute depletion of *Ddb1* by shRNA led to reduced ChREBPα without affecting the other lipogenic factor SREBP-1c (Fig. 2A). In contrast, over-expression of *Ddb1* and its binding partner *Cul4a* increased the abundance of ChREBPα but not SREBP-1c in primary mouse hepatocytes (Fig. 2B). To determine whether the interaction of ChREBPα with DDB1 is critical for the ChREBPα stability, we compared the effects of DDB1 overexpression on the full-length ChREBPα with that on the C-terminal 400-aa deletion mutant of ChREBPα (ChREBPα−400aa) in Hepa1 cells. Overexpression of DDB1 increased the abundance of the full-length ChREBPα but not the ChREBPα−400aa truncation mutant, in support of an essential role of ChREBPα-DDB1 interaction on its stability (Supplementary Fig. 5).

*Ddb*1 shRNA greatly shortened the ChREBPα half-life, whereas *Ddb* overexpression significantly increased its half-life (Fig. 2C–D). To examine how knockdown of DDB1 affects ChREBPα ubiquitination, we acutely depleted *Ddb1* in 293AD cells with Ad-shDdb1 and found such manipulation not only increased the ChREBPα ubiquitination but also decreased the ChREBPα abundance (Fig. 2E).

To further assess the impact of *Ddb1* depletion on the ChREBPα transcriptional activity, we measured the expression profile of ChREBPα targets in fructose-treated primary mouse hepatocytes with or without *Ddb1* knockdown by adenoviral shRNA. ChREBPα overexpression robustly induced the mRNA levels of *Fasn, Acc1,* and *Scd1* in primary mouse hepatocytes transduced with Ad-sh*LacZ* but not in those with Ad-shDdb1 (Fig. 2F). In summary, these results support that DDB1 likely promotes the ChREBPα stability by suppressing its ubiquitination and is required for the ChREBPα-induced lipogenic gene expression in hepatocytes.

3.3. DDB1 is required for de novo lipogenesis pathway in hepatocytes

The finding that DDB1 promotes the ChREBPα stability and ChREBP-driven lipogenic gene expression points to DDB1 as a novel regulator of hepatic lipid metabolism. To further support this notion, we observed that DDB1 was markedly increased in primary mouse hepatocytes upon 2-week high-fructose diet feeding along with the induction of FASN (Fig. 3A). Next, we examined the impact of hepatocyte Ddb1 deficiency on de novo lipogenesis. To that end, primary mouse hepatocytes from *Ddb1*$^{flox/flox}$ mice were transduced with Ad-Cre to create acute *Ddb1* deficiency (Fig. 3B). In *Ddb1* deficient hepatocytes with Ad-Cre, the mRNA levels of the classical ChREBP target genes including *Fasn* and *Scd1* were reduced almost 90%, while that of *Tnf-α* was comparable between two groups of hepatocytes (Fig. 3C). In agreement with the gene expression profile, the protein abundance of FASN, ACC1, GCK and SCD1 was also markedly reduced (Fig. 3D). More importantly, we observed a >50% reduction in the rate of de novo lipogenesis in *Ddb1*-deficient primary mouse hepatocytes after incubating the cells with the ^{3}H-labeled lipogenic substrate acetate (Fig. 3E) as well as a reduction in total TG content in those cells (Fig. 3F). In summary, our data for the first time support DDB1 as an intrinsic regulator of hepatic de novo lipogenesis pathway.

3.4. Hepatocyte-specific Ddb1 knockout mice (Ddb1-LKO) mice are resistant to high-fructose diet-induced de novo lipogenesis and liver steatosis

We hypothesize that mice with hepatocyte-deficient *Ddb1* would fail to promote lipogenic responses upon high-fructose diet due to reduced ChREBPα pathway. We therefore challenged *Ddb1*$^{flox/flox}$ with either regular chow or high-fructose diet and *Ddb1-LKO* mice with high-fructose diet for 3 weeks (Fig. 4A). By the end of experiments, there were no major differences in body weight, serum ALT, TG, and cholesterol among the three groups (Fig. 4B–E). However, liver TG was about 2-fold higher in high-fructose-fed *Ddb1*$^{flox/flox}$ mice vs. regular chow-fed group, while there was no significant difference between high fructose-fed *Ddb1*$^{flox/flox}$ and *Ddb1-LKO* groups (Fig. 4F). Loss of *Ddb1* in the liver of *Ddb1-LKO* mice was confirmed in cytosolic fractions by immunoblotting with anti-DDB1 (Fig. 4G top panel). In the nuclear fractions of the same liver lysates from *Ddb1-LKO* mice, we detected a nearly complete loss of ChREBPα (Fig. 4G bottom panel), consistent with reduced FLAG-ChREBPα in hepatocytes with acute *Ddb1* depletion (Fig. 2A).

Hepatocytes have been shown to adapt to the influx of fructose by up-regulating genes of glycolysis and de novo lipogenesis. We found that ChREBP targets such as *Fasn, Acc1, ATP-cl,* and *Scd1* were induced in the liver of high-fructose diet-fed *Ddb1*$^{flox/flox}$ mice but lost in the liver of high-fructose diet-fed *Ddb1-LKO* mice (Fig. 4H). Meanwhile, the glycolytic targets of ChREBPα such as *Gck* and *Lpk* were also reduced in high-fructose diet-fed *Ddb1-LKO* mice. In contrast, the expression of transcription factor *ChREBPα, Srebp-1c,* fatty acid elongation enzymes *(Gpat* and *Dgat),* fatty acid oxidation (FAO) enzymes as well as fructose metabolism genes in the liver were found to be similar between high-fructose diet-fed *Ddb1*$^{flox/flox}$ and *Ddb1-LKO* mice (Fig. 4I–J). This in vivo finding for the first time points to hepatic DDB1 as a fructose sensor via ChREBPα during high-fructose diet feeding.

3.5. Acute hepatocyte deficiency of Ddb1 in adult mice show impaired de novo lipogenesis and reduced liver steatosis upon high-fructose feeding

To further test the impact of *Ddb1* deficiency in adult mice on their response to high-fructose diet, we used acutely AAV-TBG-Cre to delete *Ddb1* in the liver of $Ddb1^{flox/flox}$ mice before high-fructose diet feeding (Fig. 5A). Deletion of *Ddb1* was confirmed by the absence of DDB1 in the liver with AAV-TBG-Cre injection (Fig. 5B). When compared with AAV-TBG-GFP group, AAV-TBG-Cre-injected mice on high-fructose diet showed similar body weight (Fig. 5C) but reduced serum cholesterol and TG (Fig. 5D–E). Liver weight and liver total TG content were greatly reduced in AAV-TBG-Cre-injected mice (Fig. 5F–G), in agreement with reduced lipid droplets in the liver by H&E staining (Fig. 5H). Next, we checked the protein abundance of ChREBPα in liver tissues of both groups. In line with our data with *Ddb1 -LKO* mice, the nuclear abundance of ChREBPα but not SREBP-1c was markedly reduced in the liver of AAV-TBG-Cre-injected $Ddb1^{flox/flox}$ mice (Fig. 5I). Moreover, the classical lipogenic genes such as *Fasn, Acc1,* and *ATP-cl* were reduced at both the mRNA and protein levels in the liver of those mice (Fig. 5J–K). No changes were observed with genes of FAO, lipid uptake, and VLDL secretion (Fig. 5L–M). In summary, acute deletion of *Ddb1* in adult mice also reduces the ChREBPα and mitigates high-fructose diet-induced liver steatosis.

3.6. DDB1 stabilizes ChREBPα via the suppression of CRY1-mediated ubiquitination/degradation of ChREBPα

So far we uncovered a novel role of DDB1 in protecting the ChREBP stability upon high-fructose diet. How exactly does DDB1 promote the ChREBP stability while functioning as a key component of an ubiquitin E3 ligase? We previously reported that DDB1 protects the FOXO1 protein stability by degrading CRY1 to promote hepatic gluconeogenesis [18]. We suspected that a similar mechanism might be in place to regulate the ChREBP stability. To test this possibility, we firstly tested whether the manipulation of CRY1 affects the abundance of ChREBPα. Acute knockdown of *Cry1* in both WT MEF and Hepa1 cells led to elevated ChREBPα expression (Fig. 6A & Supplementary Fig. 6A). In contrast, overexpression of *Cry1* reduced the ChREBPα abundance in both $Cry1^{-/-}Cry2^{-/-}$ MEF cells and Hepa1 cells (Fig. 6B and Supplementary Fig. 6B). Moreover, we detected the protein complex of CRY1-ChREBPα in Hepa1 cells when the proteasome activity was inhibited by MG132 (Fig. 6C). Furthermore, we observed that the polyubiquitinated ChREBPα was greatly increased in the presence of CRY1 (Fig. 6D).

We next addressed whether CRY1 is targeted for the DDB1-mediated stabilization of ChREBPα. In WT MEF cells, acutely knockdown of *Ddb1* lowered ChREBPα. However, such an effect was abrogated in WT MEF cells transduced with both Ad-shDdb1 and Ad-shCry1 (Fig. 6E). In contrast, over-expression of DDB1 blocked the negative effect of CRY1 overexpression on the ChREBPα abundance (Supplementary Fig. 7).

One of possible mechanisms by which CRY1 promotes ChREBPα degradation is via competition with DDB1 to bind to the C-terminal motif of ChREBPα. To test this hypothesis, we performed co-immunoprecipitation to examine whether a C-terminal truncation of ChREBPα impairs its binding to CRY1 in the presence of proteasome

inhibition. As shown in Supplementary Fig. 8A, the full-length ChREBPα did interact with CRY1 in hepatocyte co-transfected with constructs expressing ChREBPα and CRY1. However, the interaction was markedly reduced with both C-terminal deletion mutants (400 s and 700 s). Furthermore, overexpression of CRY1 markedly decreased the abundance of the full-length ChREBPα protein but not the 700 s mutant (Supplementary Fig. 8B). These results support that the CRY1 binding to the C-terminal motif of ChREBPα is a prerequisite for its ability to degrade ChREBPα.

Next, we examined the functional consequences of the CRY1-mediated ChREBPα degradation. Consistent with biochemical results, over-expression of either Ad-Cry1-WT or Cry1–585KA (a mutant resistant to DDB1-mediated degradation) potently suppressed the ChREBPα-mediated induction of lipogenic genes including *Fasn* and *Acc1* in primary mouse hepatocytes (Supplementary Fig. 9). Overall, our data revealed DDB1 targets CRY1 to promote the ChREBPα stability and activity in hepatocytes. Finally, we explored whether the manipulation of CRY1 affects hepatic ChREBPα -mediated de novo lipogenesis in the liver of high-fructose diet-fed mice. To raise hepatic CRY1 in WT mice on high-fructose diet, we injected WT mice with the Ad-Cry1 – 585KA mutant, which was shown to be resistant to fructose-induced down-regulation (Supplementary Fig. 10). Moreover, this approach allowed us to assess the impact of blocking DDB1 pathway on hepatic lipid metabolism from the perspective of CRY1 since we previously reported that DDB1 targets lysine 585 of CRY1 for ubiquitination-dependent degradation [19]. Adenoviral injection of CRY1–585KA during high-fructose diet feeding not only increased CRY1 levels but also altered the expression of its known targets *Dbp* and *p21* in the liver (Supplementary Fig. 11). More importantly, CRY1–585KA overexpression reduced ChREBPα in the liver of female mice after high-fructose diet feeding (Fig. 6F). Further analysis showed that the expression of de novo lipogenesis genes such as *Fasn* and *Atp-cl* were significantly reduced in the mice injected with Ad-Cry1–585KA (Fig. 6G). In the same liver tissues, the total TG was also reduced about 30% in Ad-Cry-585KA-injected mouse livers (Fig. 6H) although the difference was statically insignificant possibly due to the short duration of high-fructose diet feeding. Taken together, we presented both in vitro and in vivo evidence supporting that CRY1 negatively regulates ChREBPα protein stability and liver steatosis upon fructose feeding.

4. Discussion

As a major nutritional component, fructose intake has profound metabolic effects on lipid metabolism and potential metabolic impairments [3]. Although it has been established that ChREBP is required for fructose-induced de novo lipogenesis pathway, the exact mechanisms by which fructose stimulates de novo lipogenesis still remains poorly understood, especially the signaling pathways upstream of ChREBPα. In the current study, we discovered and confirmed DDB1 as a novel interaction protein of ChREBPα. Later, we found that DDB1 is both sufficient and necessary for stabilizing ChREBPα via CRY1. Deletion of *Ddb1* in hepatocytes not only reduces ChREBPα but also blocks hepatic de novo lipogenesis upon high-fructose diet feeding. Overexpression of degradation-resistant CRY1 mutant (585KA) leads to reduced ChREBPα and its target gene expression in the liver of mice on high-fructose diet. Therefore, the DDB1-CRY1 axis might be a novel

avenue for treating fructose-induced liver steatosis (Supplementary Fig. 12). Our findings also highlight the importance of the ubiquitination pathway in metabolic homeostasis [24]. It should be noted that the exact mechanisms by which fructose stimulates the DDB1-ChREBPα protein interaction in hepatocytes remain unknown. In our study, pharmacological inhibition of either AKT or mTOR activity was shown to reduce the amount of DDB1 interacting with ChREBPα upon fructose treatment, suggesting that the AKT-mTOR pathway might be critical for this induction by fructose. Of note, fructose feeding has been shown to increase hepatic mTOR phosphorylation in female rats [25–27]. It will be of great interest to further examine whether increased hepatic mTOR phosphorylation is required for fructose-induced DDB1-ChREBPα protein interaction and identify direct targets of mTOR in the liver during this induction.

Our finding that DDB1 promotes the ChREBPα stability appears to be counter-intuitive based on its intrinsic E3 ligase activity. We previously reported that DDB1 also enhances the stability of nuclear FOXO1 by inducing the degradation of the circadian protein CRY1 [18]. In this curent study, we discovered that CRY1 is also a potent inhibitor of the ChREBPα stability and knockdown of both *Cry1* and *Ddb1* abolishes the effects of DDB1 on ChREBPα, suggesting that CRY1 is indeed a downstream target of DDB1 to regulate the ChREBPα stability. The Lamia and Kim groups showed that CRY1 could recruit ubiquitin E3 ligases such as MDM2 and FBXL3 [28,29]. Our future work will identify the specific degradation machinery recruited by CRY1 to degrade ChREBPα. As for the functional significance of DDB1 interaction with ChREBPα, we speculate that DDB1 could compete with CRY1 for its binding to ChREBPα, therefore blocking the CRY1-mediated ChREBPα degradation. This possibility is in part supported by our finding that the ChREBPα C-terminal deletion mutant seems to be more stable than the ChREBPα-WT while failing to respond to either DDB1 or CRY1 overexpression (Supplementary Figs. 5 & 8). As such, we predict that DDB1 mutations that abolish its interaction with ChREBPα would fail to stabilize the ChREBPα. On the flip side, inhibition of CRY1 and ChREBPα interaction could enhance the ChREBPα stability. Our future study will aim to identify small compounds that might alter ChREBPα interaction with either DDB1 or CRY1 in order to manipulate the ChREBPα protein abundance.

Unexpectedly, fructose shows an opposite effect on the CRY1 stability when compared with its effect on ChREBPα (Fig. S6). In primary mouse hepatocytes, CRY1-WT became destabilized in the presence of fructose. In contrast, CRY1–585KA, a mutant resistant to DDB1-mediated degradation, remained stable, suggesting that DDB1 may target CRY1 at lysine 585 for fructose-induced CRY1 degradation. We previously reported that the DDB1-CUL4A complex acts as an E3 ligase to promote the CRY1 ubiquitination and degradation during circadian cycles [19]. Our current findings for the first time indicate this pathway is also involved in the nutrient-induced CRY1 turnover. What is still unclear is how fructose utilizes DDB1 to degrade CRY1. Fructose was found to induce the AMPK activity by depleting cellular ATP [30]. It has been reported that AMPK promotes the CRY1 degradation via direct phosphorylation [31]. Whether AMPK is required for the fructose-induced CRY1 degradation will be investigated in the future.

Given the critical role of CRY1 in the circadian clock, it would be of great interest to study the impact of fructose on the molecular circadian clock in both cells and whole animals. In fact, a study from the Froy group showed that fructose impairs the molecular circadian clock in hepatocytes while increasing the amplitude of circadian gene oscillations in muscle cells [32]. It is tantalizing to hypothesize that fructose modulates the circadian clock via the regulation of CRY1 turnover. An in-depth understanding of the mechanisms by which fructose promotes CRY1 degradation could shed light on how fructose intake affects human circadian physiology.

In summary, we identified a novel ubiquitination-dependent pathway critical for hepatic metabolism upon fructose intake. Within this pathway, elevated DDB1 degrades CRY1 to promote the ChREBPα stability and its lipogenic action in the liver in response to high-fructose diet feeding. Conversely, loss of *Ddb1* hepatocytes abrogates the lipogenic response to high-fructose diet due to ChREBPα destabilization in the liver. Our study provides a rationale for targeting DDB1 or CRY1 to treat fructose-associated metabolic impairments.

We acknowledge that our animal experiments were designed to test the acute response to fructose influx in *Ddb1*-deleted hepatocytes. Our current study does not provide detailed biochemical mechanisms in spite of the novel finding of CRY1 as a destabilizing factor for ChREBPα.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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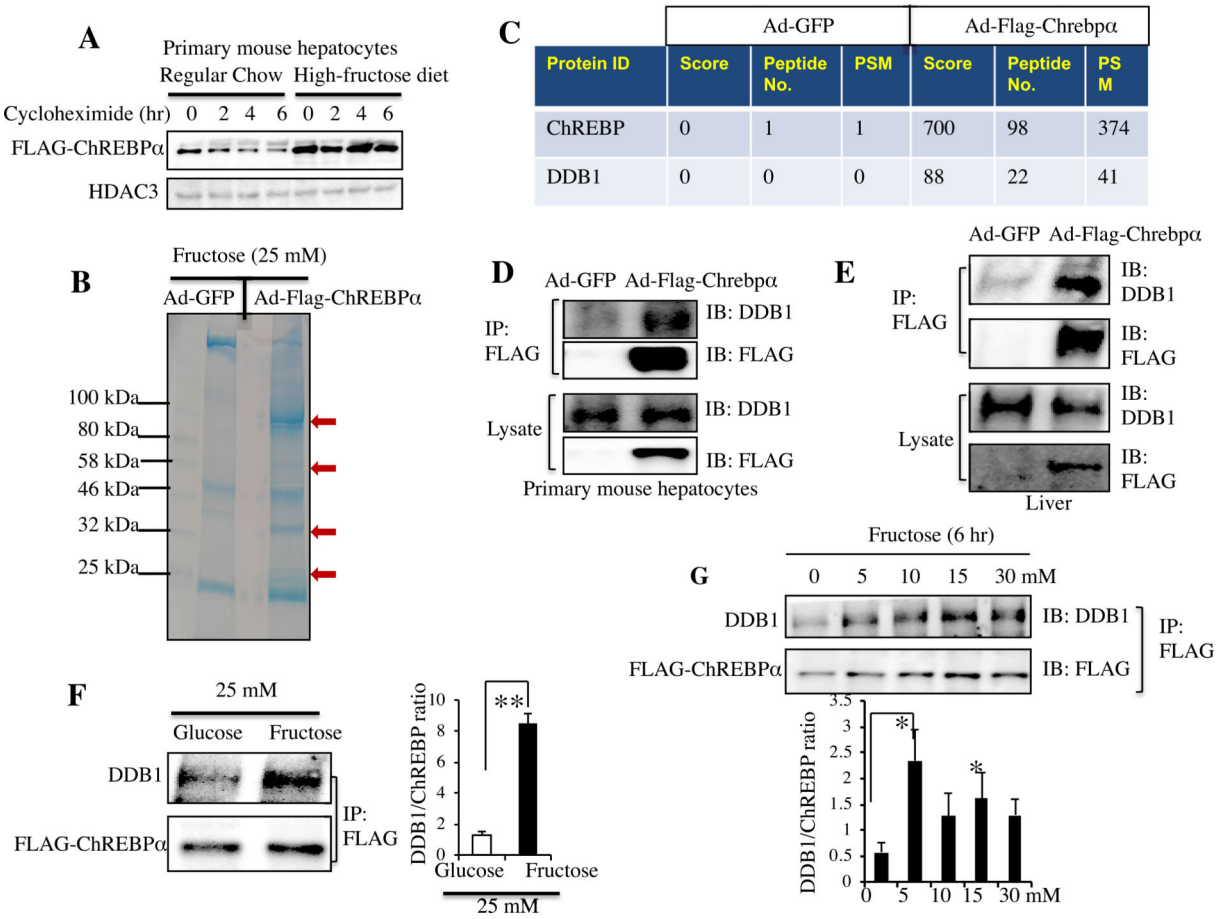

Protein ID	Ad-GFP Score	Ad-GFP Peptide No.	Ad-GFP PSM	Ad-Flag-Chrebpα Score	Ad-Flag-Chrebpα Peptide No.	Ad-Flag-Chrebpα PSM
ChREBP	0	1	1	700	98	374
DDB1	0	0	0	88	22	41

Fig. 1.

Identification of DDB1 as a novel interaction protein of ChREBP in the presence of fructose. (A) Fructose increases the ChREBP stability in primary mouse hepatocytes. Primary mouse hepatocytes were transduced with Ad-Flag-Chrebpα and exposed to fructose (25 mM) for 16 h before treatment of cycloheximide for indicated time points. (B) 293A cells were transduced with Ad-Flag- Chrebpα or Ad-GFP for 16 h and incubated in medium containing 25 mM fructose. The FLAG-ChREBPα complex was purified by immunoprecipitation with FLAG-M2 beads, separated on SDS-PAGE gel, and stained with Colloidal Blue Staining. Four bands (indicated by red arrows) were excised out for MS. (C) The partial list of ChREBPα-interacting proteins. PSM: peptide spectrum matches. DDB1 protein was identified. (D) Detection of FLAG-ChREBPα interaction with the endogenous DDB1 in primary mouse hepatocytes. (E) Detection of FLAG- ChREBPα interaction with the endogenous DDB1 in mouse liver tissue. After injection with Ad-GFP or Ad-Flag-Chrebpα, mouse livers were subjected to IP with anti-FLAG M2 beads and then IB with anti-DDB1. (F) Effects of glucose vs. fructose on ChREBPα protein interaction with DDB1 in primary mouse hepatocytes. The quantification of three experiments was shown on right. (G) Dose-dependent effects of fructose on ChREBPα and DDB1 protein interaction in primary mouse hepatocytes. The quantification of three experiments was shown on right. Data were plotted as mean ± SD (n = 3). * < 0.05 by the Students-*t*-test (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

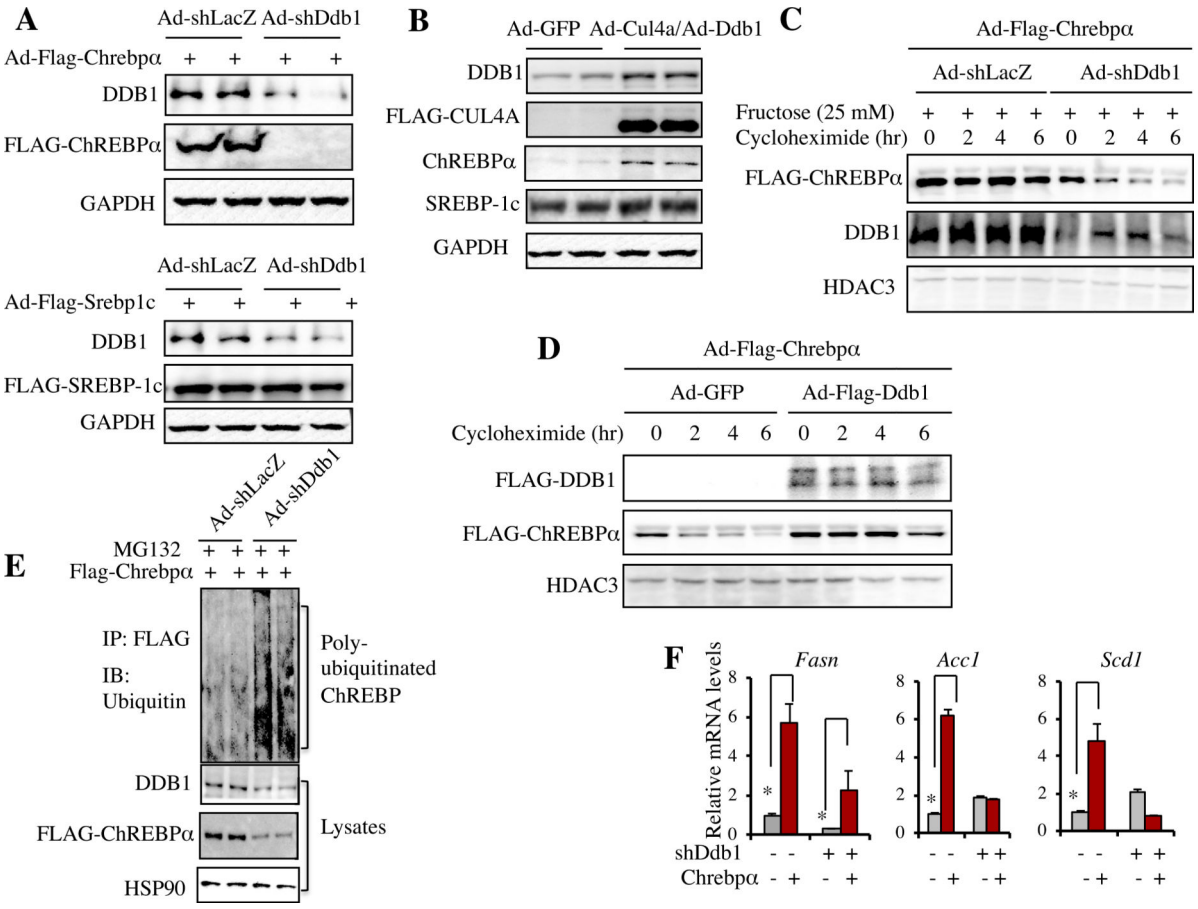

Fig. 2.

DDB1 promotes ChREBPα protein stability in hepatocytes. (A) Effects of acute *Ddb1* knockdown on the protein abundance of ChREBPα and SREBP-1c. Primary mouse hepatocytes were firstly transduced with Ad-shLacZ and Ad-shDdb1 and then transduced with equal amount of Ad-Flag-Chrebpα or Ad-Flag-Srebp-1c. The cells were maintained in the medium with l0 mM fructose and harvested 24 h later. The protein lysates were used to examine the abundance of FLAG-ChREBPα and FLAG-SREBP-1c. (B) Effects of acute DDB1 overexpression on the protein abundance of ChREBPα or SREBP-1c. Primary mouse hepatocytes were transduced with either Ad-GFP or a combination of Ad-Ddb1 and Ad-Cul4a. The cells were maintained in regular medium and harvested 24 h later for Western blot to detect the abundance of ChREBPα and SREBP-1c. (C) Acute *Ddb1* knockdown on ChREBPα proteins stability upon cycloheximide. The protein stability of FLAG-ChREBPα increased in primary mouse hepatocytes transduced with Ad-shDdb1 and cultured in fructose containing medium. (D) Acute *Ddb1* overexpression extends ChREBPα protein half-life in primary mouse hepatocytes in the absence of fructose. (E) Acute Ddb1 knockdown increases ChREBPα ubiquitination in hepatocytes cultured in the presence of fructose. (F) Effects of *Ddb1* knockdown on ChREBPα-driven expression of lipogenic genes in fructose-treated primary mouse hepatocytes. The data were presented as mean ± SD. *$p < 0.05$.

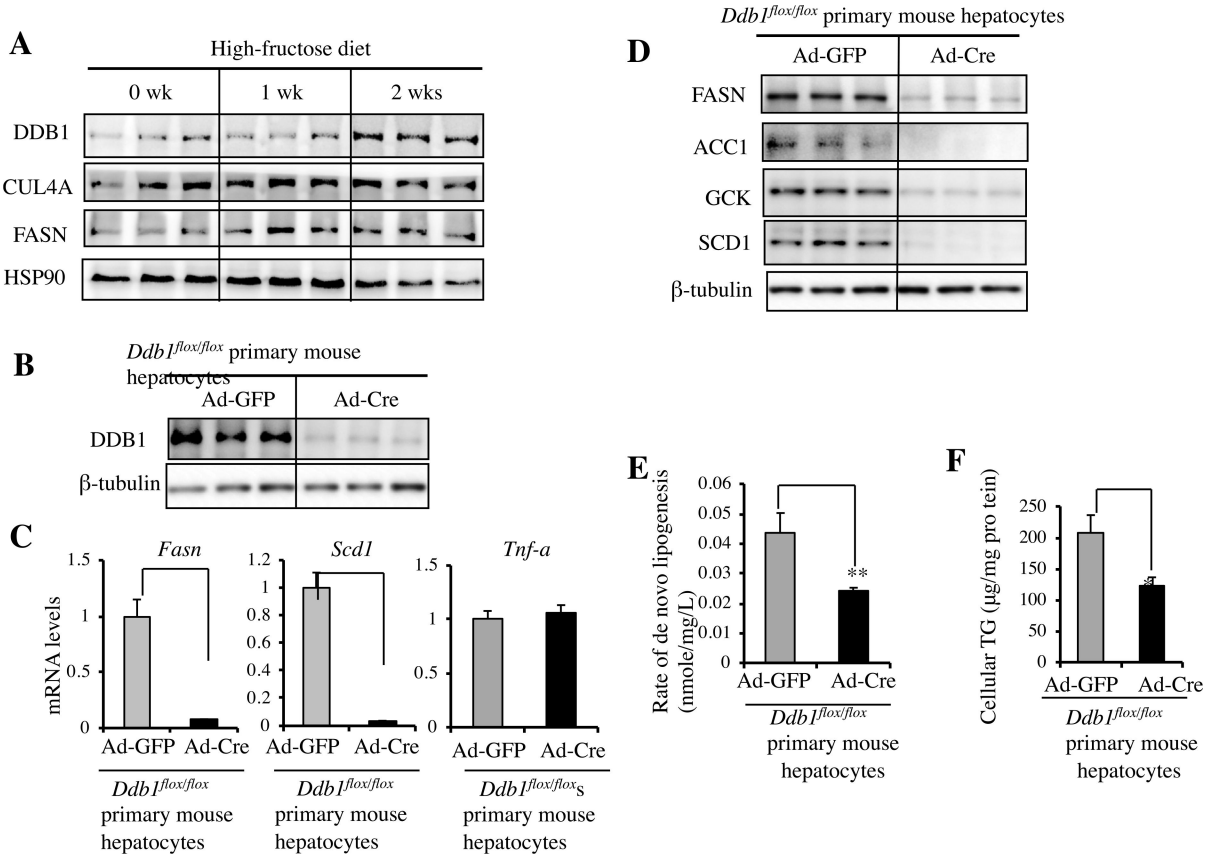

Fig. 3.

DDB1 is an essential regulator of de novo lipogenesis in hepatocytes. (A) Elevated DDB1 protein in primary mouse hepatocytes isolated from mice on regular chow, 1-week high-fructose diet, or 2-week high-fructose diet. Induction of lipogenic enzyme FASN was also detected by immunoblotting. (B) Generation of acute *Ddb1*-deficient hepatocytes by transduction of *Ddb1*$^{flox/flox}$ primary mouse hepatocytes with Ad-Cre. Confirmation of *Ddb1* deletion by immunoblotting; (C) *Ddb1*-deficient primary mouse hepatocytes show reduced mRNA levels of lipogenic genes including *Fasn* and *Scd1.* (D) *Ddb1*-deficient primary mouse hepatocytes show reduced protein expression of lipogenic proteins; (E-F) *Ddb1-deficient* primary mouse hepatocytes show reduced rate of de novo lipogenesis and triglyceride content. The data were presented as mean ± SD. *$p < 0.05$ and **$p < 0.01$.

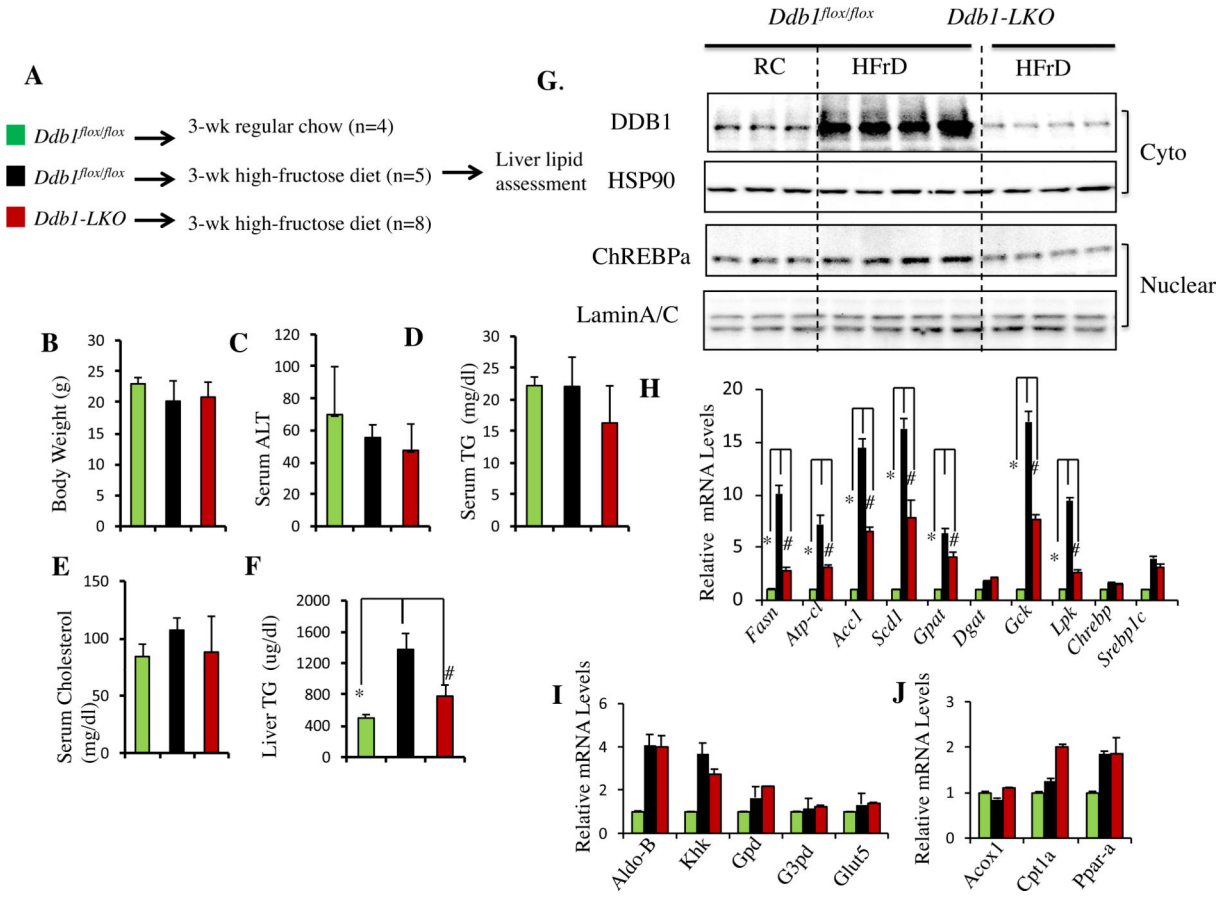

Fig. 4.

Ddb1-LKO mice are resistant to short-term high-fructose diet-induced lipogenesis in liver. (A) Schematic of animal experiments on regular chow or high-fructose diet (n = 4 for *Ddb*$^{flox/flof(flox)}$*-RC*; n = 5 for *Ddb1*$^{flox/flox}$-HFrD; n = 8 for *Ddb1-LKO*-HFrD); (B-H) body weight, serum ALT, serum TG, serum cholesterol, and liver TG were measured among these groups; (I) representative immunoblotting of hepatic DDB1 and ChREBPα protein in chow-fed and high-fructose diet-fed *Ddb1*$^{flox/flox}$, as well as high-fructose diet-fed *Ddb1-LKO.* ((I&J) The mRNA levels for enzymes in de novo lipogenesis, fructolysis, and fatty acid oxidation in three experimental groups. **Ddb1*$^{flox/flox}$-regular chow vs. *Ddb1*$^{flox/flox}$-high-fructose diet; #*Ddb1*$^{flox/flox}$-high-fructose diet vs. *Ddb1-LKO*-high-fructose diet

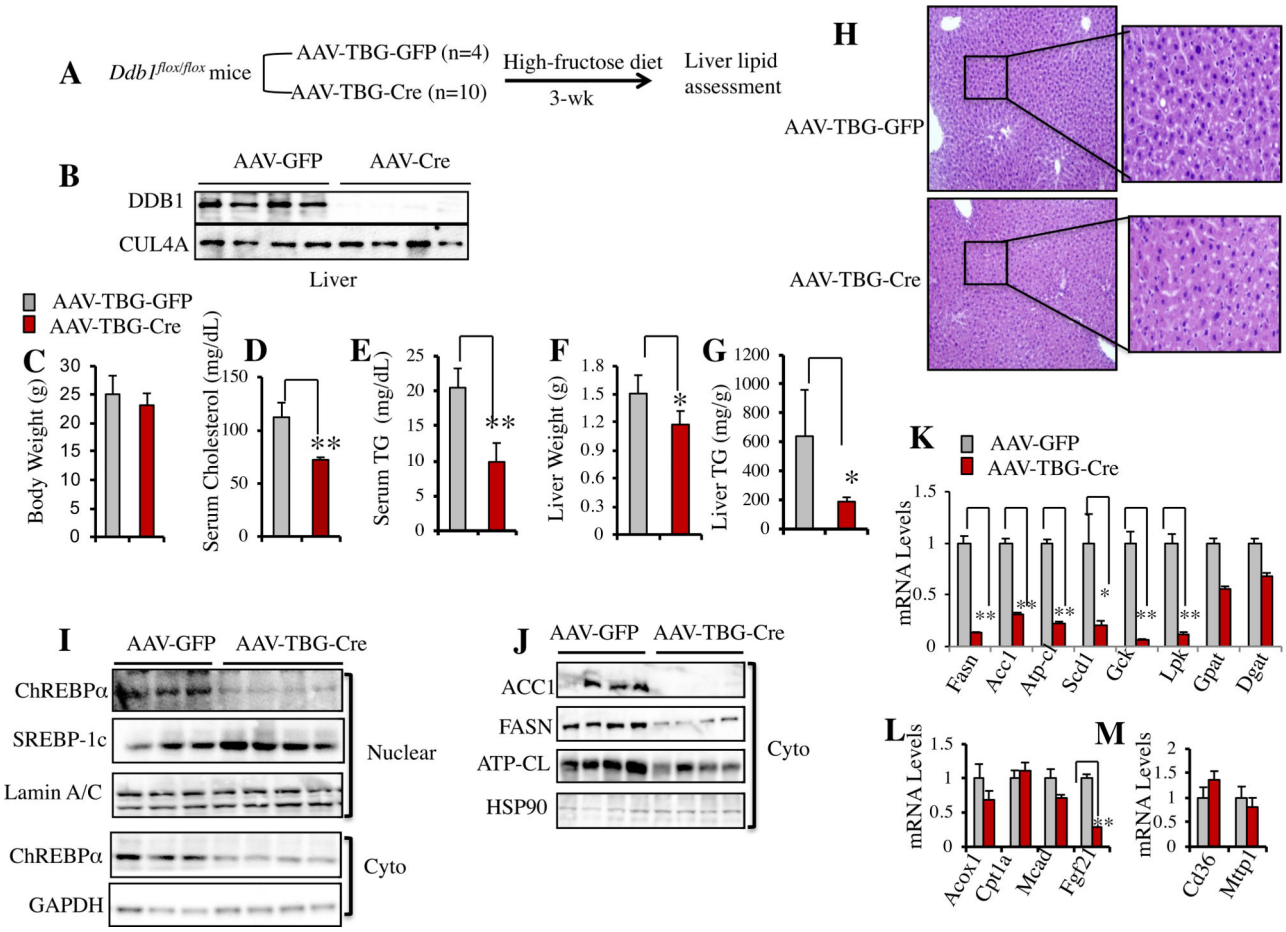

Fig. 5.

Acute deletion of hepatic *Ddb1* in adult mice blocks liver metabolic response to high-fructose diet feeding. (A) Generation of adult-onset-hepatocyte *Ddb1* knockout mouse model. (B) Absence of DDB1 protein in the liver of AAV-TBG-Cre-injected mice by immunoblotting; (C–G) BW, serum TG, cholesterol, liver weight and liver TG between AAV-GFP and AAV-Cre mice; (H) representative of liver histology by HE staining; (I–J) protein abundance of cytosolic/nuclear ChREBPα, nuclear SREBP-1c, and cytosolic lipogenic enzymes in liver lysates from AAV-GFP and AAV-Cre groups; (K–M) the mRNA expression of genes in lipid metabolism in liver cDNA prepared from both groups of mice. $*p < 0.05$ and $**p < 0.01$ by unpaired *t*-test.

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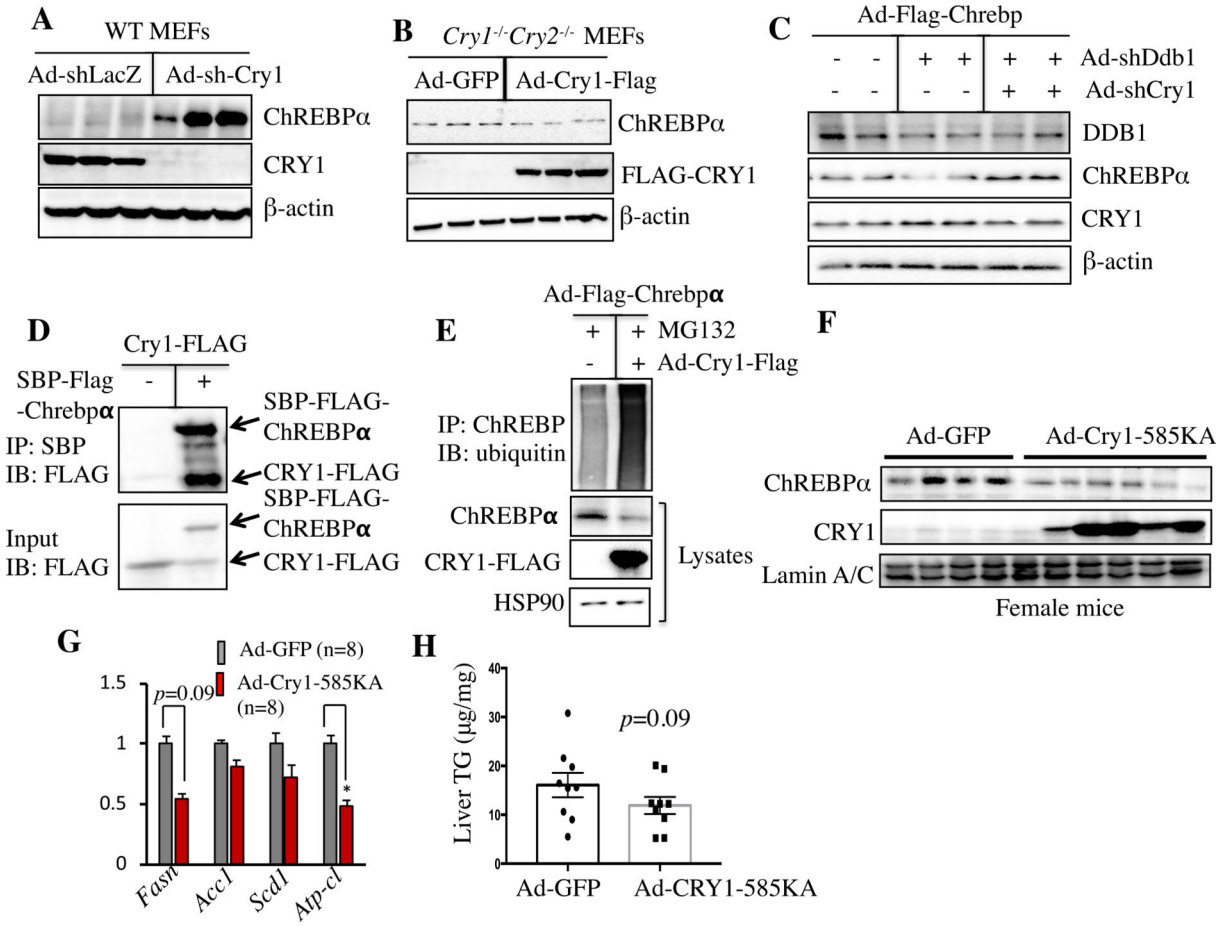

Fig. 6.

CRY1 mediates DDB1-dependent ChREBPα protein stabilization. (A) Acute knockdown of *Cry1* by Ad-shCry1 in WT-MEF increases ChREBPα protein expression. (B) Restoring CRY1 in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MEFs by Ad-Cry1 reduces ChREBPα protein; (C) effects of *Ddb1* knockdown or *Ddb1/Cry1* double knockdown on ChREBPα protein in primary mouse hepatocytes; (D) detecting protein-protein interaction between CRY1 and ChREBPα. 293A cells were transfected with SBP-CBP-ChREBPα and Cry1-Flag and protein lysates were used in immunoprecipitation with Anti-SBP beads and the presence of CRY1 was detected by anti-FLAG; (E) overexpression of CRY1 increases ChREBPα ubiquitination in primary mouse hepatocytes. (G) The protein abundance of both ChREBPα and CRY1 in the liver of WT mice injected with Ad-GFP or Ad-Cry1–585KA. (H) The mRNA expression of genes in lipid biosynthesis in the liver from Ad-GFP- or Ad-Cry1–585KA-injected of mice. *$p <$ 0.05. (I) Liver TG between Ad-GFP and Ad-Cry1–585KA groups.