DDB1-mediated CRY1 degradation promotes FOXO1-driven gluconeogenesis in liver

Running title: DDB1 promotes gluconeogenesis by degrading CRY1

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ABSTRACT

Targeted protein degradation through ubiquitination is an important step in the regulation of glucose metabolism. Here we present evidence that the DDB1-CUL4A ubiquitin E3 ligase functions as a novel metabolic regulator that promotes FOXO1-driven hepatic gluconeogenesis. In vivo, hepatocyte-specific Ddb1 deletion leads to impaired hepatic gluconeogenesis in the mouse liver but protects mice from high-fat diet (HFD)-induced hyperglycemia. Lack of Ddb1 downregulates FOXO1 protein expression and impairs FOXO1-driven gluconeogenic response. Mechanistically, we discovered that DDB1 enhances FOXO1 protein stability via degrading the circadian protein cryptochrome 1 (CRY1), a known target of DDB1 E3 ligase. In the Cry1 depletion condition, insulin fails to reduce the nuclear FOXO1 abundance and suppress gluconeogenic gene expression. Chronic depletion of Cry1 in the mouse liver not only increases FOXO1 protein but also enhances hepatic gluconeogenesis. Thus, we have identified the DDB1-mediated CRY1 degradation as an important target of insulin action on glucose homeostasis.
INTRODUCTION

Maintaining steady levels of glucose is crucial for the survival and health of mammals during feeding and fasting cycles. During fasting, the liver serves as the major site to keep blood glucose steady by activating de novo gluconeogenesis. During feeding, insulin reduces the glucose surge by promoting glycogen synthesis while inhibiting gluconeogenesis in the liver (1-3). The hepatic insulin signaling cascade has been shown to be indispensable in maintaining glucose homeostasis in animal models. Mice lacking insulin receptors in hepatocytes quickly develop hyperglycemia and hyperinsulinemia independently of obesity (4). In diabetic patients, severe hepatic insulin resistance has been considered as a major driver in excessive hepatic glucose production and fasting-associated hyperglycemia (2). Therefore, the ability of insulin to suppress hepatic glucose production has been targeted to treat diabetes.

Previous studies have established FOXO1 as a key transcription factor in integrating insulin signaling and hepatic glucose metabolism (5; 6). Under normal physiological conditions, insulin activates the PI3K-AKT pathway, which in turn phosphorylates the transcription factor FOXO1 at T24/S253/S316 (7). Subsequently, phosphorylated FOXO1 is excluded from the nucleus, leading to reduced mRNA levels of key gluconeogenic enzymes such as G6pase (glucose-6-phosphatase) and Pepck (phosphoenolpyruvate carboxykinase)(8-11). However, in the case of insulin resistance, the nuclear accumulation of FOXO1 promotes transcription of gluconeogenic enzymes and eventually results in hyperglycemia (12; 13). FOXO1 has been found to be targeted for proteasomal degradation via ubiquitination downstream of insulin signaling (14-16). Several E3 ligases including MDM2, SKP2, and COP1 promote FOXO1 ubiquitination and degradation at least in vitro (17-19). Since inactivation of FOXO1 transcriptional activity is the major route
for insulin to inhibit gluconeogenesis, inhibition of FOXO1-mediated gluconeogenesis by targeting its degradation could offer a promising avenue to treat diabetes without activating the insulin-AKT-dependent lipogenic pathway.

DDB1 (DNA-damage binding protein 1) is a scaffolding component of the DDB1-CUL4A ubiquitin E3 ligase complex (20-22). Within this complex, DDB1 serves as the linker protein between CUL4A and substrate-binding proteins (21; 23). Ddb1 deletion disrupts the DDB1-CUL4A complex and subsequently abolishes its E3 ligase actions (21; 24; 25). DDB1-CUL4A E3 ligase promotes ubiquitination and degradation of a variety of substrates, including p27, c-JUN, CDT1 (23; 26; 27). We recently discovered that DDB1-CUL4A E3 ligase targets CRY1 for ubiquitination and degradation via DCAF protein CDT2 (28). Ddb1 deletion increases total levels of CRY1 protein in mouse hepatocytes and mouse liver. So far, the role of DDB1 in liver metabolism is largely unknown since Ddb1 global knockout is embryonically lethal (24). Given the emerging role of CRY1 in liver metabolism (29; 30), it is likely that DDB1 could function as a metabolic regulator at least by manipulating the CRY1 protein stability.

CRY1 is an evolutionarily conserved clock protein with diverse functions (31). Besides its classical function as a negative regulator of the circadian network, CRY1 has been shown to be a novel regulator of glucose metabolism. Ectopic over-expression of CRY1 in liver reverses hyperglycemia in db/db mice, a type 2 diabetes model, by interfering with CREB-dependent glucagon signaling (30). CRY1 was also found to modulate glucocorticoid receptor function during the induction of gluconeogenesis (29). Recently, SREBP-1c-induced Cry1 has been
shown to regulate hepatic glucose production (32). All these studies have highlighted the critical role of CRY1 in hepatic glucose metabolism under both normal and pathological conditions.

In this study, we examined the role of DDB1 E3 ligase in hepatic glucose metabolism. We established for the first time that DDB1 E3 ligase is a novel positive regulator of hepatic gluconeogenesis. Hepatocyte-deficiency of Ddb1 not only suppresses hepatic gluconeogenesis during fasting but also protects mice from HFD-induced fasting hyperglycemia. At the mechanistic level, DDB1 increases FOXO1 protein via CRY1 degradation and promotes the FOXO1-driven gluconeogenesis in hepatocytes. In Cry1-depleted hepatocytes, insulin fails to reduce nuclear FOXO1 abundance and repress gluconeogenic gene expression. Therefore, our study discovered a novel pathway to regulate the FOXO1-driven gluconeogenesis via DDB1-mediated CRY1 degradation.

**RESEARCH DESIGN AND METHODS**

**Animals**

Animal experiments were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee of University of Michigan Medical School. Male C57BL/6J mice and Albumin-Cre mice were purchased from the Jackson Laboratory. The Ddb1floxflox mice were backcrossed to the C57BL/6J background for at least nine generations. The liver-specific Ddb1 knockout (Ddb1-LKO) mice were generated by crossing Ddb1floxflox mice with Albumin-Cre mice. All mice were housed on a 12hr:12hr light/dark cycle at 25 °C with free access to water and regular chow or high-fat diet (45% kcal from fat, Research Diets). For fasting experiments, mice were fasted for 16 hr from 6 pm to 10 am.
Metabolic parameter measurements

Levels of blood glucose were measured with a Contour glucometer (Bayer). Aliquots of serum were analyzed for insulin levels by an insulin ELISA kit (R&D Systems). Pyruvate tolerance tests (PTT) were performed in fasting animals at the indicated time points after intra-peritoneal injection of sodium pyruvate in saline at 2 g/kg.

RNA isolation and RT-qPCR

The RNA isolation and RT-qPCR analysis were performed as described previously (28). The primer sequences used in qPCR analysis were listed in the supplementary material. All gene expression experiments were performed in at least two independent experiments in biological triplicates.

Protein extraction, immunoprecipitation, and ubiquitination assay

To prepare cytosolic and nuclear proteins, liver tissues or cell pellets were homogenized in hypotonic buffer, incubated on ice for 15-20 min, and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was saved as cytosolic fraction. The pellet was washed once with hypotonic buffer and re-suspended in RIPA buffer prior to sonication for 5 seconds. The nuclear protein was then collected after centrifugation at 13000 rpm x 10 min. Immunoprecipitation protocol to detect protein-protein interaction has been described previously (28). FLAG-M2 beads or streptavidin beads were added into the lysate to capture immunocomplex containing FLAG-CRY1 or CBP-CRY1. The protocol for detecting protein ubiquitination has been described previously (33). Anti-FOXO1 was used to pull down FOXO1-ubiquitin conjugates.
Proximity Ligation Assay

PLA was performed using Duolink In Situ PLA reagent according to the instructions from Sigma. After overnight transduction with Ad-Foxo1 and Ad-Cry1 in chamber slides, $Cry1^{-/-}Cry2^{-/-}$ MEF cells were fixed in 4% paraformaldehyde at 4 °C for 10 min. After quenching with 0.1 M glycine and permeabilization with 0.25% Triton-X 100, cells were blocked in 10% BSA and then incubated in 1/250 diluted primary antibody solutions (anti-FOXO1 H-128 (SC-11350) anti-CRY1 W-L5 (SC-101006), both from Santa Cruz). Afterwards, the slide was incubated in PLA mixture of Duolink In Situ Probe anti-Rabbit PLUS and Duolink In Situ Probe anti-Mouse MINUS in a pre-heated humidity chamber at 37 °C for 1 hr. After ligation and amplification at 37 °C with Duolink In Situ Detection Reagents Far Red, the slide was mounted with cover slips using Duolink In Situe Mounting Medium with DAPI. Imaging was performed on a Zeiss Axio Imager M2 microscope with filters set at $\lambda_{Ex} = 360$ nm/$\lambda_{Em} = 460$ nm for DAPI and at $\lambda_{Ex} = 647$ nm/$\lambda_{Em} = 669$ nm for far red. Nine individual fields under 63x oil objective were captured for each condition and quantified using the ImageJ software.

Cell cultures, transfection, and treatments

Both 293T and Hepa1c1c-7 cells were purchased from ATCC and maintained according to the instructions. Transient transfection in 293T or Hepa1 cells was performed using polyethyleneimine (28). Primary mouse hepatocyte isolation was described previously (28). WT mouse embryonic fibroblasts (MEF) and $Cry1/2$ double knockout MEF were generously provided by Dr. John Hogenesch (University of Cincinnati). Adenoviral transductions in cells were performed using 1.0 x $10^8$ plaque-forming units (pfu) per well of 6-well plates for 16 hr. To assay FOXO1 transcriptional activity, 293T cells in a 24-well plate were transfected with the
G6Pase-luc construct (contains 1.2 kb promoter sequence upstream transcription starting site) alongside various combinations of expression vectors. 36 hr post transfection, cells were lysed for luciferase activity measurement on a BioTek Synergy 2 microplate reader. A β–galactosidase construct was co-transfected in each well for normalization of luciferase activity.

**Generation and injection of recombinant adenoviruses**

Adenoviruses including Ad-shLacZ, Ad-shDb1, Ad-Flag-Cry1-WT and Ad-Flag-Cry1-585KA, and Ad-Ddb1 were described (28). Ad-FOXO1-ADA was generously provided by Dr. Henry Dong at the University of Pittsburg. Ad-Myc-Foxo1-WT was generated using Gateway technology described before (28). AAV7TBG-CRE and AAV7TBG-GFP were purchased from the UPENN Vector core. For adenoviral injections, 1x10^{12} pfu per adenovirus were administrated via tail-vein injection. For each virus, a group of 4 to 5 mice were injected with the same dose of viral particles. 10-14 days days after injection, mice were sacrificed at ZT8 after overnight fasting and liver tissues were harvested for protein analysis.

**Western blot**

Western blot analysis was performed using the following primary antibodies: anti-DDB1 (Abcam), anti-CRY1 (sc-101006), anti-GAPDH (sc-25778), anti-Lamin A/C (sc-20681), anti-PEPCK (sc-32879), anti-G6PASE (sc-25840), anti-FOXO1 (sc-11350), anti-CUL4A (sc-10782), anti-AKT1/2 (sc-1619), anti-CBP (sc-33000) (Santa Cruz biotechnology), anti-phospho-AKT (S473), anti-phospho-GSK-3β (S9) from (Cell Signaling Technology), anti-ubiquitin and anti-β-tubulin (Sigma-Aldrich). Anti-p85 was a gift from Dr. Liangyou Rui at the University of Michigan.
**Statistical analysis**

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

**RESULTS**

**Hepatocyte-deletion of Ddb1 impairs glucose metabolism in regular chow-fed mice.**

So far, the metabolic actions of DDB1 in the liver have never been tested. We set out to investigate whether Ddb1 deficiency in hepatocytes could impact glucose metabolism in mice. We firstly confirmed Ddb1 deletion in the Ddb1-LKO mouse liver and primary mouse hepatocytes by measuring DDB1 protein expression (Fig. 1A and Supplementary Fig. 1A). The residual DDB1 signal in Ddb1-LKO mouse liver was likely to be from other cell types within the liver since the Ddb1 mRNA is ubiquitously expressed (Supplementary Fig. 1B). In the same liver tissues, we observed increased levels of CRY1 protein (Fig. 1A), consistent with our previous report (28). Hepatocyte deletion of Ddb1 reduced blood glucose levels during fasting and refeeding without affecting body weight (Fig. 1B-E), suggesting DDB1 protein could act as a physiological regulator of glucose metabolism. The liver maintains steady levels of blood glucose during fasting by enhancing hepatic gluconeogenesis (34). To assess the role of DDB1 in this process, we performed pyruvate tolerance tests. Compared with Ddb1\textsuperscript{floox/floox} mice, Ddb1-LKO mice showed impaired hepatic gluconeogenic response after gluconeogenic substrate pyruvate was injected (Fig. 1F). This reduction in gluconeogenic response is consistent with lowered protein and mRNA levels of PEPCK and G6Pase, two key enzymes in gluconeogenesis.
These data, for the first time, demonstrated DDB1 as a new metabolic regulator of hepatic glucose metabolism.

**Hepatocyte-deletion of Ddb1 protects mice from HFD-induced hyperglycemia and curbs hepatic gluconeogenesis**

During the development of insulin resistance and type-2 diabetes caused by high-fat diet, unchecked gluconeogenesis in the liver is a major contributor to fasting hyperglycemia (35; 36). Given the inhibitory effects of Ddb1 deficiency on hepatic gluconeogenesis (Fig. 1F-H), we asked whether hepatic DDB1 level is affected by high-fat feeding. As shown in Fig. 2A, 12-wk HFD (45% calories from fat) markedly elevated the protein level of DDB1 in the mouse liver without affecting its binding partner CUL4A (Fig. 2A). This seems to be a liver-specific phenomenon since DDB1 protein expression remained similar in adipose tissues from the same cohort of mice fed either regular chow (RC) or HFD (Fig. 2A). Moreover, induction of liver DDB1 protein by HFD possibly occurs through post-translational modifications since its mRNA levels are comparable between RC and HFD group (Supplementary Fig 2A-B). Taken together, these studies suggest that the DDB1 protein abundance is sensitive to nutrient status in hepatocytes.

To test whether deletion of hepatic Ddb1 could impact gluconeogenesis in mouse liver following HFD feeding, both Ddb1^floxflox^ and Ddb1-LKO mice were challenged with HFD for 12 wks. Compared with Ddb1^floxflox^ mice fed HFD for 6-12 weeks, Ddb1-LKO mice displayed similar body weight gain (Fig. 2B) but lower levels of fasting glucose (Fig. 2C). Furthermore, Ddb1-LKO mice showed suppressed hepatic glucose production in PTT tests after HFD feeding.
after 4 weeks of HFD feeding (Fig. 2D). Consistent with reduced glucose production in the liver, the mRNA and protein levels of G6Pase and PEPCK were reduced in Ddb1-LKO mice (Fig. 2E-F). Meanwhile, the nuclear CRY1 protein was enhanced in Ddb1-LKO mice (Fig. 2F). Taken together, our data suggested that hepatic Ddb1 deficiency reduces blood glucose and suppresses hepatic gluconeogenesis during the course of HFD. Of note, hepatic Ddb1 deficiency shows no impact on systemic glucose utilization (measured by GTT) and insulin sensitivity (by ITT) after chronic HFD treatment (Supplementary Fig. 3).

**Acute hepatocyte Ddb1 deficiency in adult mice impairs HFD-induced hepatic gluconeogenesis**

To further test how hepatocyte DDB1 expression affects gluconeogenic response in adult mice, we used AAV-Cre to delete hepatic Ddb1 in 8-wk Ddb1lox/lox mice and then subjected to HFD feeding for another 6 weeks (Fig. 3A). Acute Ddb1 deletion did not affect body weight (Fig. 3B), but severely blunted hepatic gluconeogenesis response upon pyruvate tolerance test (Fig. 3C). Even after 6 hr fasting we observed that mice injected with AAV-Cre showed higher blood glucose (134 mg/dL) in comparison with mice with AAV-GFP (77 mg/dL). Moreover, we detected significant reduction in G6pase and Pepck mRNA and protein expression in the liver of HFD-treated AAV-Cre-injected Ddb1lox/lox mice (Fig. 3D-E). These data suggest that hepatic DDB1 in adult mice plays a crucial role in elevating hepatic glucose production when challenged by HFD.

Hepatic gluconeogenesis is mainly driven by transcription activators such as FOXO1, CREB, GR, and HNF-4α (2; 3). It is possible that Ddb1 deficiency could lead to impaired abilities of
these transcription factors to promote gluconeogenesis. We focused on the potential role of FOXO1 because a recent study identified CRY1 as a suppressor of FOXO1 downstream of SREBP-1c (32). In a luciferase assay, Ddb1 deficiency repressed the induction of the G6pase promoter-driven luciferase activity by Foxo1, whereas DDB1 overexpression augmented the luciferase activity (Supplemental Fig. 4). Although DDB1 functions as a scaffolding protein in the DDB1-CUL4A-CDT2 E3 ligase complex, we observed a great reduction in nuclear FOXO1 protein in Ddb1-deleted liver or hepatocytes (Fig. 3F-G). Moreover, inhibition of proteasomes by MG132 blocked the FOXO1 reduction caused by Ddb1 depletion (Fig. 3H). These results suggest that DDB1 may control FOXO1 protein stability, in particularly nuclear FOXO1 abundance, to regulate gluconeogenic response.

**CRY1 mediates the DDB1 effects on FOXO1 stabilization**

Since DDB1 serves as a linker protein in the CUL4A ubiquitin E3 ligase complex, it is counterintuitive that Ddb1 deficiency reduces FOXO1 protein abundance in our study. We speculated that this regulation could be mediated through one of the DDB1 substrates. We reported that the DDB1-CUL4A E3 ligase targets CRY1 for ubiquitination-dependent degradation to regulate the molecular clock activity and CRY1 level is increased in the Ddb1-deficient mouse liver (28). CRY1 was also shown to be a negative regulator of hepatic gluconeogenesis mediated by GR, CREB, and most recently FOXO1 (29; 30; 32). To test whether CRY1 protein might be a likely candidate to mediate FOXO1 degradation, we firstly compared the overall levels of the endogenous FOXO1 in WT vs. Cry1−/−/Cry2−/− MEF cells. Indeed, total FOXO1 protein expression is inversely correlated to total CRY1 protein (Fig. 4A). Next, we compared the nuclear abundance of FOXO1 protein in WT vs. Cry1−/−/Cry2−/− MEF cells
since it is the nuclear FOXO1 that drives the transcription of its target genes. The nuclear FOXO1 was also markedly increased in Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF (Fig. 4B). Conversely, restoring CRY1 expression in Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF was sufficient to reduce the nuclear FOXO1 abundance (Fig. 4B). It is known that nuclear FOXO1 abundance is influenced by either stability and/or localization. To distinguish these possibilities, we performed a similar experiment using the mutant FOXO1 (FOXO1-ADA) that is constitutively localized in the nucleus due to the loss of AKT-dependent phosphorylation (7). Similar to FOXO1-WT, the levels of FOXO1-ADA were also increased in the Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF (Fig. 4C). Taken together, our data suggest that CRY1 down-regulates mainly the nuclear FOXO1 protein abundance.

To directly address whether CRY1 could be a downstream mediator linking DDB1 and FOXO1, we performed acute knockdown of Ddb1 in Ad-Foxo1-transduced WT and Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF cells. In agreement with AAV-Cre-transduced Ddb1<sup>fl/fl</sup> mouse liver and PMHs (Fig. 3F-G), Ddb1 depletion reduced the nuclear FOXO1 protein in WT MEF but not in Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF (Fig. 4D). Conversely, over-expression of DDB1 increased nuclear FOXO1 in WT MEF but not in Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF (Fig. 4E). These data support that DDB1 promotes the nuclear FOXO1 abundance via degrading CRY1.

**CRY1 interacts with FOXO1 and promotes its ubiquitination and degradation**

To further explore how CRY1 regulates FOXO1 protein turnover, we measured levels of poly-ubiquitinated FOXO1 protein in WT or Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF cells by immunoblotting with anti-ubiquitin after IP with anti-FOXO1. Ubiquitination of FOXO1 was greatly reduced in Cry1<sup>−/−</sup>/Cry2<sup>−/−</sup> MEF (Fig. 5A). In contrast, Cry1 over-expression enhanced the formation of
polyubiquitinated FOXO1 in WT MEF cells (Fig. 5B). These data suggest that CRY1 down-regulates FOXO1 protein stability by promoting its ubiquitination and degradation.

How does CRY1 promote FOXO1 ubiquitination and degradation? It is possible that CRY1 could interact with FOXO1 and recruit its E3 ligase. Indeed, a strong interaction of CRY1 was detected with either FOXO1-WT or FOXO1-ADA in co-transfected 293T cells and transduced PMHs (Fig. 5C-E). The protein interaction between CRY1 and FOXO1 in the nucleus was further validated by a proximity ligation assay with anti-FOXO1 and anti-CRY1 in Cry1−/−/Cry2−/− MEF cells transduced with both Ad-Cry1 and Ad-Foxo1 (Fig. 5F). Furthermore, such interaction was found to require the CRY1-terminal region (300-614aa) after domain mapping via a series of CRY1 truncation mutants (Supplementary Fig. 6).

**Cry1 depletion abrogates insulin-induced suppression of FOXO1 activity**

Upon food intake, insulin suppresses FOXO1 transcription via InsR-AKT signaling (5; 7). Whether CRY1 contributes to insulin-induced suppression of FOXO1 action has not been tested yet. We observed that CRY1 protein in primary hepatocytes was quickly induced within 1-hr treatment with insulin (Fig. 6A). Such regulation was also observed in the fasted mouse livers 2 hr after insulin injection (Fig. 6B). In both cases, the Cry1 mRNA remained unchanged (Supplementary Fig. 7) within 2 hours of insulin treatment, suggesting that insulin is likely to promote CRY1 protein stabilization independently of transcriptional activation during the first 2 hr insulin exposure.
Next, we asked whether CRY1 could affect insulin induced nuclear FOXO1 protein degradation in mouse hepatocytes (15; 16). Consistent with the literature (6; 7), insulin treatment reduces the nuclear FOXO1 in Ad-shLacZ-transduced PMHs. However, its effect was abolished in the Ad-shCry1-transduced PMHs (Fig. 6C), suggesting that CRY1 is required for insulin-induced nuclear FOXO1 degradation. To further test the role of CRY1 in insulin suppression of gluconeogenesis, we compared the mRNA levels of G6Pase and Pepck in PMHs transduced with Ad-shCry1 vs. Ad-shLacZ. As shown in Fig. 6E-F, insulin potently suppresses mRNA of G6Pase and Pepck in dose-dependent manner in cells transduced with Ad-shLacZ but not in cells transduced with Ad-shCry1. Intriguingly, Pepck mRNA was significantly increased in cells transduced with Ad-shCry1 after insulin treatment. Thus, CRY1 is required for insulin-induced suppression of nuclear FOXO1 and gluconeogenic gene expression in hepatocytes.

**Acute depletion of Cry1 in mice leads to elevated fasting glucose and gluconeogenic response**

To further evaluate the impact of Cry1 depletion on glucose metabolism in vivo, we injected WT mice with Ad-shCry1 vs. Ad-shLacZ via tail-vein. The CRY1 protein was measured in the mouse liver 14 days post injection. CRY1 protein levels were reduced in Ad-shCry1-injected liver, whereas FOXO1 protein was elevated (Fig. 7A). 7 days following injection, we detected a significant increase in blood glucose level in Ad-shCry1-injected mice after overnight fasting compared with Ad-shLacZ-injected mice (Fig. 7B), a phenotype similar to that in Cry1 and Cry2 double knockout mice (29; 37). To assess the gluconeogenic activity in the Cry1-depleted liver, we performed PTT in both groups of mice at 7 days post-injection. Consistently, we observed a significant increase in blood glucose in response to pyruvate in mice injected with Ad-shCry1.
(Fig. 7C). The serum insulin levels were slightly reduced in the shCry1 group without statistical significance (Fig. 7D). Meanwhile, the levels of *G6Pase*, *Pepck* and *Pgc-1α* (co-activator for gluconeogenesis) were significantly induced (Fig. 7E). Taken together, our data support CRY1 as a negative regulator of hepatic gluconeogenesis in vivo.

*Cry1* and *Cry2* double knockout mice develop systemic insulin resistance when challenged with HFD (38). However, the tissue-specific role of CRY1 in insulin sensitivity remains unclear. To gain insights into the impact of chronic *Cry1* depletion on liver insulin signaling, we examined the phosphorylation levels of downstream targets of insulin in the Ad-shCry1-injected liver after 2-wk HFD feeding. Consistent with elevated gluconeogenesis, the levels of AKT-P^S473^ and GSK-3β-P^S9^ were reduced in the liver with acute *Cry1* knockdown in HFD-fed mice (Fig. 7F), suggesting that chronic *Cry1* depletion could lead to impairment of insulin-AKT signaling which further promotes FOXO1 accumulation in the liver. To confirm this indeed occurs in hepatocytes, we isolated PMH from mice injected with shCry1 for more than 7 days and found these hepatocytes were also very resistant to insulin-stimulated AKT-P^S473^ (Supplementary Fig. 8).

To gain insights into how chronic *Cry1* deficiency may lead to insulin resistance in hepatocytes. We performed RT-qPCR analysis of known genes associated with insulin resistance in mice injected with either Ad-shLacZ or after HFD feeding. In both liver and PMHs of Ad-shCry1-injected mice, the expression of the known negative regulator of insulin signaling *Socs3* was significantly increased (Supplementary Fig. 9A-B) (39; 40). Induction of *Dbp* mRNA was used as a marker for *Cry1* depletion. Since CRY1 has been also implicated in inhibiting inflammation
in various tissues (41; 42) and chronic inflammation has been linked to insulin resistance (43; 44), we checked the expression of several pro-inflammatory markers in the liver of Ad-shCry1-injected mice. As shown in Supplementary Fig. 9C, hepatic Cry1 depletion increased the expression of Tnfa and Mcp-1 in the liver of Ad-shCry1-injected mice. Taken together, our data showed that acute Cry1 deficiency results in hepatic insulin resistance by up-regulating the pathways that impede insulin signaling and subsequently exacerbate the FOXO1-driven gluconeogenesis.

**DISCUSSION**

Uncontrolled hepatic glucose production is a hallmark of type 2 diabetes (2; 35). Here, we uncovered a novel metabolic pathway in which DDB1 E3 ligase promotes FOXO1-driven hepatic gluconeogenesis by degrading CRY1. Furthermore, deletion of Ddb1 in hepatocytes protects mice from HFD-induced fasting hyperglycemia and elevated gluconeogenesis without affecting body weight. In addition, we demonstrated that insulin induces CRY1 protein and CRY1 depletion impairs insulin’s ability to suppress FOXO1-mediated gluconeogenesis. In conclusion, our study discovered DDB1-mediated CRY1 degradation as a novel pathway to regulate FOXO1 protein expression and gluconeogenesis in the liver (Fig 7G). Given that a specific small molecule activator of CRY1 has been shown to repress glucagon-induced gluconeogenesis in PMHs (45), suppression of DDB1 E3 ligase-mediated ubiquitination and degradation of CRY1 may offer another therapeutic avenue to control hyperglycemia in type 2 diabetics.
Targeted protein ubiquitination and degradation have been shown to regulate cell cycle, genomic stability, and DNA replication (46; 47). How protein ubiquitination regulates metabolic events is far less understood. Emerging evidence suggests that E3 ligases could be important regulators of glucose metabolism (48). For example, the ubiquitin E3 ligase MG53 promotes IRS-1 ubiquitination and degradation and therefore contributes to obesity and metabolic syndrome upon chronic high fat diet (49; 50). In contrast, the ubiquitin E3 ligase CBL-B promotes ubiquitination and degradation of TLR4 and therefore reduces macrophage activation and infiltration during obesity (51). Collectively, these findings suggest that E3 ligases could be effectively targeted to restore metabolic homeostasis. In our current study, we provide both in vivo and in vitro evidence suggesting an important role of DDB1-CUL4A E3 ligase in hepatic glucose metabolism. *Ddb1* deficiency represses FOXO1-driven gluconeogenesis in hepatocytes and in turn reduces blood glucose during fasting. Interestingly, DDB1 protein is elevated in the mouse liver following HFD, raising the possibility that induction of DDB1 might be required to protect FOXO1 stability during insulin resistance. Since CRY1 has also been implicated to suppress the cAMP-CREB and glucocorticoid-mediated gluconeogenesis pathway (29; 30), it remains to be tested whether DDB1 could also regulate these two pathways in addition to FOXO1 in both fasting and HFD conditions.

We provide the first evidence that insulin induces CRY1 protein expression independently of gene expression in both hepatocytes and liver. We propose two possible mechanisms that may account for such an acute action of insulin on CRY1 stability. The first possibility is that the insulin-AKT signaling pathway promotes phosphorylation of CRY1 to block its interaction with DDB1-CUL4A-CDT2 E3 ligase, therefore protecting CRY1 from degradation. However,
sequence analysis by Scansite revealed that CRY1 contains no canonical phosphorylation motifs for AKT. The second possibility is that insulin can block the formation of DDB1-CUL4A-CDT2 E3 ligase. Two recent reports highlighted a role of FBOX11 E3 ligase in promoting CDT2 ubiquitination and degradation in response to TGF-β signaling (52; 53), implying a possibility of signal-dependent CDT2 protein degradation. Sequence analysis suggests that CDT2 is a preferred substrate for a number of kinases including AKT, GSK-3β, ATM, and DNA-PK. Thus, CDT2 could be a potential direct target of AKT to modulate DDB1-CUL4A E3 ligase activity. More detailed biochemical analysis will be needed to determine whether insulin inhibits DDB1-CUL4A-CDT2 E3 ligase assembly and activity via direct phosphorylation.

Unexpectedly, we observed that acute Cry1 deficiency leads to a marked reduction in AKT activation in both liver and primary mouse hepatocytes. This effect might be due to a combination of induction of both negative regulator of insulin signaling (SOCS3) and pro-inflammatory markers. How CRY1 is involved in suppressing these pathways remains to be addressed. These findings also suggest that CRY1 can inhibit FOXO1 signaling via two distinctive mechanisms in a time-dependent manner: on one hand, CRY1 acutely binds to nuclear FOXO to promote its ubiquitination-dependent degradation. On the other hand, chronic activation of CRY1 could enhance AKT signaling and therefore facilitates the translocation of FOXO1 to the nucleus to further inhibit gluconeogenic gene expression.

In conclusion, our study has identified an intricate mechanism of how DDB1 stabilizes FOXO1 through CRY1 degradation during fasting. Our study highlighted the critical role of DDB1 in promoting FOXO-1-dependent gluconeogenesis in the liver. Given the function of DDB1 in the
CUL4A E3 ligase complex, it is conceivable that targeting DDB1 might offer novel therapeutics for the treatment of type 2 diabetes.

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Author contributions: X.T. and L.Y. designed and performed in vitro experiments. X.T. and L.Y. wrote and edited the manuscript. N.C., K.D., D.Z. and X.T. conducted in vivo experiments, E. J., N. G., and K.S. carried out RT-qPCR and the generation of adenoviral constructs and concentration of adenoviruses. J. S. analyzed and quantified the PLA imaging data. L.Y. supervised the work, analyzed and interpreted the data. L.Y. is the sole guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Figure legends

Figure 1. Loss of Ddb1 in the liver impairs hepatic glucose metabolism in chow-fed mice. Ddb1<sup>flax/flax</sup> and Ddb1-LKO male mice (6-8 wk) on regular chow feeding were compared in terms of body weight and blood glucose after either 12-hr fasting or 8-hr refeeding. (A) Immunoblotting of DDB1 and CRY1 in the liver of Ddb1<sup>flax/flax</sup> and Ddb1-LKO mice (n = 3). (B-C) Body weight and blood glucose in Ddb1<sup>flax/flax</sup> (n = 7) and Ddb1-LKO (n = 4) mice after 16-hr fasting. (D-E) Body weight and blood glucose in Ddb1<sup>flax/flax</sup> (n=7) and Ddb1-LKO (n = 8) mice 8 hrs post refeeding after 16-hr fasting. (F) Pyruvate tolerance test in Ddb1<sup>flax/flax</sup> (n = 8) and Ddb1-LKO (n = 6) mice following 16-hr fasting. (G-H) Protein and mRNA levels of G6Pase and Pepck in the liver of Ddb1<sup>flax/flax</sup> and Ddb1-LKO after 16-hr fasting. * denotes p-value < 0.05. Data were plotted as mean ± S.D..

Figure 2. Hepatic deletion of Ddb1 protects mice from HFD-induced hyperglycemia and gluconeogenesis. (A) DDB1 and CUL4A protein in the liver and white adipose tissue of WT mice on 12-wk regular chow vs. HFD feeding. (B-C) Body weight and fasting glucose of Ddb1<sup>flax/flax</sup> (n = 4) vs. Ddb1-LKO (n = 5) during 12-wk HFD; (D) Pyruvate tolerance test after 4-week HFD feeding in Ddb1<sup>flax/flax</sup> (n = 8) vs. Ddb1-LKO mice (n = 7) and AUC analysis; (E) The mRNA levels of G6pase and Pepck in the liver of Ddb1<sup>flax/flax</sup> (n = 8) vs. Ddb1-LKO (n = 7) after 12-wk HFD; (F) Cytosolic levels of DDB1, CRY1, G6Pase, and PEPCK as well as nuclear CRY1 in the liver of Ddb1<sup>flax/flax</sup> vs. Ddb1-LKO mice after 12-wk HFD. Data were presented as mean ± S.D.. * p-value < 0.05 and ** p-value < 0.01.
Figure 3. Acute adult-onset hepatic Ddb1 deficiency impairs HFD-induced gluconeogenesis.
(A) Generation of acute adult-onset deletion of Ddb1 in the liver by tail vein injection of Ddb1^floxfloxflox mice with AAV-TBG-Cre for PTT after 6-wk HFD. The control group mice were injected with AAV-TBG-GFP. (B) Body weight of Ddb1^floxfloxflox mice injected with either AAV-GFP (n = 6) or AAV-Cre (n = 8) before and after HFD; (C) Pyruvate tolerance test after 6-week HFD in AAV-GFP vs. AAV-Cre group mice and AUC analysis; (D) The mRNA levels of G6pase and Pepck in the liver of AAV-GFP vs. AAV-Cre group mice after 7-wk HFD; (E-F) Cytosolic levels of DDB1, G6Pase, and PEPCK and nuclear FOXO1 protein in the liver of AAV-GFP vs. AAV-Cre group mice after 7-wk HFD; (G) Nuclear FOXO1 protein in the Ddb1^floxfloxflox PMHs transduced with AAV-GFP vs. AAV-Cre. Cytosolic levels of DDB1, G6Pase, and PEPCK were also examined by immunoblotting; (H) Rescue of FOXO1 protein expression by proteasome inhibitor MG132 in Ad-shDdb1-transduced Hepa1 cells. Hepa1 cells were firstly transduced with Ad-Foxo1 in addition to either Ad-shLacZ or Ad-shDdb1. 48 hr later cells were treated with or without MG132 (10 µM) for 8 hr before harvest. Protein levels of DDB1, FOXO1 and GAPDH were examined by immunoblotting.

Figure 4. DDB1 represses FOXO1 through CRY1. (A) Levels of total FOXO1 protein in the WT and Cry1^-/-Cry2^-/- MEF cells transduced with Ad-Cry1. Cells were harvested 36 hrs post adenoviral transduction. (B) Restoring CRY1 expression reduces nuclear FOXO1 protein in Cry1^-/-Cry2^-/- MEF cells. WT and Cry1^-/-Cry2^-/- MEF cells were transduced with Ad-Foxo1 plus either Ad-GFP or Ad-Cry1. 36 hrs post transduction, FOXO1 protein in the nuclear extracts was detected by Western blot. (C) Accumulation of FOXO1-ADA protein in the Cry1^-/-Cry2^-/- null MEF. WT vs. Cry1^-/-Cry2^-/- MEF were transduced with Ad-Foxo1-ADA. 36 hrs post
transduction, FOXO1-ADA protein in the nuclear extracts was detected by Western blot. (D) Acute Ddb1 depletion reduces the nuclear abundance of FOXO1 protein in WT MEF but not Cry1−/−Cry2−/− MEF cells. Both WT and Cry1−/−Cry2−/− MEF cells were firstly transduced with Ad-shLacZ or Ad-shDdb1 for 6 hrs prior to transduction with Ad-Foxo1. Nuclear FOXO1 protein was detected by Western blot 36 hrs post transduction. (E) Effects of DDB1 over-expression on nuclear FOXO1 in WT and Cry1−/−Cry2−/− MEF cells. Both WT and Cry1−/−Cry2−/− MEF cells were first transduced with Ad-GFP or Ad-Ddb1 6 hr prior to transduction with Ad-Foxo1. Nuclear FOXO1 protein was detected by Western blot 36 hrs post transduction.

Figure 5. CRY1 interacts with FOXO1 and promotes its ubiquitination. (A) Reduced FOXO1 ubiquitination in Cry1/2 null MEF cells. Both WT and Cry1−/−Cry2−/− MEF were transduced with Ad-FOXO1 and treated with MG132 for 4 hr. The denatured cell lysates were subjected to immunoprecipitation (IP) with anti-FOXO1 and then immunoblotting with anti-ubiquitin. (B) CRY1 overexpression increases FOXO1 ubiquitination in Cry1−/−Cry2−/− MEF cells. Cry1−/−Cry2−/− MEF cells were transduced with Ad-GFP vs. Ad-Cry1 for 6 hrs and transduced again with Ad-Foxo1. After treatment with MG132 for 4 hrs, the denatured cell lysates were subjected to IP with anti-FOXO1 and then immunoblotting with anti-ubiquitin to examine FOXO1 ubiquitination. (C-D) CRY1 interacts with FOXO1 WT or FOXO1-ADA in 293T cells. 293T cells were co-transfected with Foxo1 plus NTAP-alone or NTAP-Cry1. After pull-down with streptavidin beads, the CRY complex was subjected to immunoblotting with anti-FOXO1 and anti-CBP. (E) CRY1 interacts with FOXO1 in PMHs. After transduction with Ad-Foxo1 plus Ad-GFP or Ad-Cry1, PMHs were subjected to IP with anti-FLAG and Western blot for FOXO1 and FLAG-CRY1. (F) CRY1 interaction with FOXO1 in the nucleus by PLA.
After transduction with Ad-Foxo1 and Ad-Cry1, Cry1−/−Cry2−/− MEF cells were incubated with both anti-CRY1 and anti-FOXO1 antibodies and subjected to PLA with Dualink In Situ PLA probes. No antibody and single antibody incubation were included as negative controls. Data were presented as mean ± S.D.. *p-value < 0.05 and **p-value < 0.01.

Figure 6. CRY1 is required for insulin-induced suppression of gluconeogenesis. (A) Insulin increases CRY1 protein levels in primary mouse hepatocytes. The endogenous CRY1 protein in PMHs was detected by Western blot following insulin treatment at 10 nM for the indicated durations. The number refers to the fold induction of CRY1 protein after normalization to the level of β-tubulin. Both total FOXO1 and nuclear FOXO1 protein were measured as well. (B) Insulin increases CRY1 protein levels but reduces nuclear FOXO1 protein in the mouse liver. WT mice were fasted for 6 hrs prior to intra-peritoneal injection of insulin at 1 U/kg for 2 hr. Hepatic CRY1 was detected by Western blot and its fold of induction by insulin was calculated after normalization to β-tubulin. Both total FOXO1 and nuclear FOXO1 protein were measured as well. (C) Depletion of Cry1 impairs insulin action on nuclear FOXO1 protein. PMHs were transduced with Ad-shLacZ or Ad-shCry1 for 24 hrs and transduced by Ad-Foxo1 for additional 24 hrs. PMHs were serum starved for 16 hrs and then stimulated with insulin at 10 nM for 2 hrs. Nuclear abundance of FOXO1 and CRY1 was examined by immunoblotting. (D & E) Cry1 knockdown abrogates insulin actions on the G6pase and Pepck expression. PMHs were transduced with Ad-shLacZ or Ad-shCry1 for 24 hrs and serum-starved overnight prior to insulin treatment and RT-qPCR analysis. Data were presented as mean ± S.D.. *p-value < 0.05.
Figure 7. Chronic depletion of CRY1 promotes hepatic gluconeogenesis. WT mice were injected Ad-shLacZ vs. Ad-shCry1 via tail vein and examined as follows: (A) levels of CRY1 and FOXO1 protein in the liver by Western blot 14 days after injection. Nuclear FOXO1 protein in the same liver tissues was also detected and quantified by immunoblotting. (B) non-fasting and fasting blood glucose levels 10 days after injection; (C) PTT test 7 days after injection. (D) Serum levels of insulin after 6-hrs fasting 14 days after injection; (E) RT-qPCR analysis for the mRNA expression of G6pase, Pepck, and Pgc-1α in the liver 14 day after injection. (F) AKT and GSK3β phosphorylation levels in the liver 14 days after injection. The ratio of AKT-P^{S473} / AKT and GSK3β-P/GSK3β were calculated. Data were presented as mean ± S.D.. * p-value < 0.05 and ** p-value < 0.01. (G) The schematics of DDB1 regulation of hepatic gluconeogenesis through CRY1. In the absence of insulin, DDB1-CUL4A-CDT2 E3 ligase ubiquitinates CRY1 for proteasomal degradation. Decreased CRY1 leads to stabilization of FOXO1 and thus promotes gluconeogenesis. In Ddb1-deficient hepatocytes, CRY1 stabilization results in constant degradation of FOXO1 and reduced gluconeogenesis.

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Figure 3

A. \(Ddb1^{lox/lox}\) mice AAV-GFP \(\xrightarrow{\text{HFD}}\) AAV-Cre \(\xrightarrow{6\text{-wk} PTT}\)

B. Body weight

C. Pyruvate tolerance test

D. Relative mRNA levels

E. Relative protein levels

F. Expression (FOXO1/Lamin A/C)

G. DDB1, PEPCK, G6Pase, GAPDH, FOXO1, Lamin A/C

H. MG132, Ad-Foxo1, Ad-shLacZ, Ad-shDdb1, DDB1, FOXO1, GAPDH

Figure 3-TIFF
**Figure 4**

### A

|          | WT  | Cry1−/−/Cry2−/− |
|----------|-----|----------------|
| Ad-GFP   | +   | +             |
| Ad-Cry1  | -   | -             |
| FOXO1    |     |               |
| CRY1     |     |               |
| GAPDH    |     |               |

### B

|          | Cytosolic | Nuclear |
|----------|-----------|---------|
| Ad-Foxol | + + +     | + + +   |
| Ad-GFP   | - + -     | - + -   |
| Ad-Cry1  | + - +     | + - +   |
| FOXO1    |           |         |
| CRY1     |           |         |
| Lamin A/C|           |         |
| GAPDH    |           |         |

### C

|          | WT  | Cry1−/−/Cry2−/− |
|----------|-----|----------------|
| Ad-GFP   | +   | -             |
| Ad-Foxol-ADA | -   | +             |
| FOXO1    |     |               |
| CRY1     |     |               |
| Lamin A/C|     |               |

### D

|          | WT  | Cry1−/−/Cry2−/− |
|----------|-----|----------------|
| Ad-Foxo1 | +   | +             |
| Ad-shDdb1| -   | +             |
| FOXO1    |     |               |
| CRY1     |     |               |
| DDB1     |     |               |
| Lamin A/C|     |               |

### E

|          | WT  | Cry1−/−/Cry2−/− |
|----------|-----|----------------|
| Ad-GFP   | +   | +             |
| Ad-Ddb1  | -   | +             |
| Ad-Foxo1 | +   | +             |
| DDB1     |     |               |
| FOXO1    |     |               |
| CRY1     |     |               |
| Lamin A/C|     |               |
Figure 5

A  IP: NoAb  FOXO1
Cryl/2  WT -/-  WT -/-
Ad-Foxo1  + + + +

IB: Ubiquitin
FOXO1  
CRY1  
GAPDH  

FOXO1-ubiquitin conjugates

B  IP: NoAb  FOXO1
Ad-Cry1  - + - +
Ad-Foxo1  + + + +

IB: Ubiquitin
FOXO1  
CRY1  
GAPDH  

FOXO1-ubiquitin conjugates

C  MG132  + +
NTAP  + -
NTAP-CRY1  - +
FOXO1  + +

FOXO1  
CBP-CRY1  

Pull-down: Streptavidin

WCL

D  Input  IP
Ad-Foxo1-ADA  + + + +
- - - -

NTAP-Cry1  
IP: anti-SBP
IB: anti-FOXO1
IP: anti-SBP
IB: anti-CBP

E  Ad-Foxo1  + +
Ad-Cry1-Flag  - +

FOXO1  
CRY1-FLAG  

IP: FLAG

WCL

F  Blank  Anti-FOXO1

Anti-CRY1  Anti-FOXO1 + anti-CRY1

Intensity (AU)

Figure 5-TIFF
Figure 6

A

PMHs

|        | Insulin (10 nM) |
|--------|-----------------|
| 0      |                 |
| 0.5    |                 |
| 1      |                 |
| 2 hr   |                 |

CRY1

AKT-P\(S^{473}\)

AKT

FOXO1

\(\beta\)-tubulin

FOXO1

HDAC3

B

Liver

PBS

Insulin

CRY1

AKT-P\(S^{473}\)

AKT

FOXO1

\(\beta\)-tubulin

FOXO1

Lamin A/C

Expression (CRY1/\(\beta\)-tubulin)

Expression (FOXO1/Lamin A/C)

C

Ad-shLacZ  Ad-shCRY1

Insulin

- +

- +

FOXO1

Lamin A/C

CRY1

AKT-P\(S^{473}\)

AKT

D

Relative \(G_{0}pase\) mRNA

Ad-shLacZ  Ad-shCRY1

Insulin 0 nM

Insulin 1 nM

Insulin 10 nM

* * *

E

Relative \(Pegck\) mRNA

Ad-shLacZ  Ad-shCRY1

Insulin-0 nM

Insulin-1 nM

Insulin-10 nM

* *
Figure 7

A) Western blot analysis showing the expression levels of CRY1, FOXO1, and p85 in Lysate and Nuclear compartments for Ad-shLacZ and Ad-shCry1 groups.

B) Blood glucose levels in non-fasting and fasting conditions for Ad-shLacZ (n=8) and Ad-shCry1 (n=10).

C) Blood glucose levels and AUC (AUIC) over time for Ad-shLacZ (n=8) and Ad-shCry1 (n=7).

D) Serum insulin levels for Ad-shLacZ and Ad-shCry1.

E) mRNA levels for G6Pase, Pepck, and Pgc-1α.

F) Western blot analysis for AKT-phosphorylated at Ser473 (AKT-Ps473), total AKT (AKT), GSK-3β-phosphorylated at Ser9 (GSK-3β-Ps9), total GSK-3β (GSK-3β), and β-tubulin.

G) Schematic diagram illustrating the pathways involving Insulin, HFD, DDB1-CUL4A E3 ligase, CRY1 stabilization, FOXO1 degradation, and Hepatic gluconeogenesis.
Supplementary Information

Mouse qPCR primers used in this study

| Gene   | Forward                      | Reverse                      |
|--------|------------------------------|------------------------------|
| G6Pase | 5’-ccggttttgacacgtcatct-3’   | 5’-caatgcctgacaaagactca-3’   |
| Pepck  | 5’-actcgagttcgcacccct-3’     | 5’-aggcagcatcaatgatggg-3’    |
| Pgc-1α | 5’-tgacaggaagcaatttttca-3’   | 5’-ttacctgcgaagctctct-3’     |
| Ddb1   | 5’-gtcctgatgatggccct-3’      | 5’-cccctgaggatcctggag-3’     |
| Cry1   | 5’-cgagttgcgtgtatgagc-3’     | 5’-atagacgcagcgatgtg-3’      |
| Dbp    | 5’-ggaactgaacctcaaccaat-3’   | 5’-ctccggctcagactttct-3’     |
| Socs3  | 5’-ccctgcagcccctccttcac-3’   | 5’-gcccccacccagccccatcc-3’   |
| Pten   | 5’-tgatacgacatgaactgctt-3’   | 5’-gctgggtcatatgtct-3’       |
| Pib1b  | 5’-ccgagagctacagagat-3’      | 5’-aaagggcctggtgagaata-3’    |
| Ship   | 5’-tgctcagcatccgaaggt-3’     | 5’-tgctctctcagctcatca-3’     |
| Shp-2  | 5’-actggcctgtgatgacat-3’     | 5’-tgctrtctcttcctgaccc-3’    |
| Rictor | 5’-cgaatacgagggcctagta-3’    | 5’-cagatggccagcttctca-3’     |
| Mcp1   | 5’-atggagcatctctgtgttg-3’    | 5’-cctgttctcagctgg-3’        |
| Il1β   | 5’-aagagcttccagcagcagatca-3’ | 5’-tgccagctgtctaggaac-3’     |
| Il6    | 5’-gccaacttggccatgg-3’       | 5’-atgcaggggtgtaatgtg-3’     |
| Tnfa   | 5’-ccagcggtgccctagtc-3’      | 5’-cagccacttccagctc-3’       |

Supplementary Figure Legends
Supplementary Figure 1. DDB1 expression in hepatocytes from Ddb1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> and Ddb1-LKO mice. (A) Absence of DDB1 protein in primary hepatocytes isolated from Ddb1-LKO mice vs Ddb1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice. The levels of β-tubulin was examined as a loading control. (B) Tissue-specific mRNA expression of Ddb1 in WT mice (n = 3). The data were plotted as mean ± SD (n = 3).

Supplementary Figure 2. High fat diet shows no effects on Ddb1 mRNA levels in both the liver and white adipose tissue (WAT). (A) Ddb1 mRNA in the liver; (B) Ddb1 mRNA in WAT. The data were plotted as mean ± S.D. (n = 5/group).

Supplementary Figure 3. Both Ddb1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> and Ddb1-LKO mice were treated with HFD and subjected to GTT and ITT test at indicated times after HFD feeding. The data were plotted as mean ± SEM. (n = 4-5).

Supplementary Figure 4. Effects of Ddb1 manipulation on the FOXO1 transcriptional activity. (A) 293T cells were transfected with Foxo1 and G6Pase-luc in the presence of either shLacZ or shDdb1. 48 h later, the luciferase activities were measured and normalized by β-gal activity. (B) 293T cells were transfected with expression vectors of Foxo1 and G6Pase-luc in the presence of GFP or Ddb1 plus Cul4a. 48 h later, the luciferase activities were measured and normalized by β-gal activity. The data were plotted as mean ± S.D. (n=3). * p-value < 0.05 and ** p-value < 0.01. The experiment was repeated at least 3 times.
Supplementary Figure 5. CRY1 suppresses the FOXO1-driven transcription activity. (A) 293T cells were transfected with vectors expressing either GFP or CRY1-WT along with expression vectors with Foxo1 and G6pase-luc. (B) 293T cells were transfected with vectors expressing either GFP or CRY1-WT along with expression vectors with Foxo1-3A (a constitutive-active form of FOXO1) and G6pase-luc. 48 h later, the luciferase activities were measured and normalized by internal control b-gal. The data were plotted as mean ± S.D. (n=3). p-value ** < 0.01 was determined by two-tailed Student’s t-test. The experiment was repeated at least 3 times.

Supplementary Figure 6. Mapping the region of CRY1 protein for its interaction with FOXO1. (A) Schematics of CRY1 C-terminal truncation mutants. (B) 293T cells were transfected with vectors encoding Cry1-wt and Cry1 truncation mutants along with Myc-Foxo1. 36 hr later cells were harvested for immunoprecipitation assay with anti-FLAG agarose beads. The presence of Myc-FOXO1 and CRY1-FLAG protein was detected by western blot with anti-Myc and anti-FLAG.

Supplementary Figure 7. Effects of acute insulin treatment on the Cry1 mRNA in PMHs and mouse liver. The mRNA was extracted from PMHs treated with 10 nM of insulin or from the mouse liver 2 hr after intra-peritoneal injection with insulin for indicated time points. The Cry1 mRNA level was measured by RT-qPCR analysis. The data were plotted as mean ± S.D. (n = 5-8).
Supplementary Figure 8. Chronic Cry1 depletion reduces hepatocyte response to insulin. (A) Schematic diagram of experiment design; (B) Primary hepatocytes infected with Ad-shCry1 vs Ad-shLacZ were exposed to insulin (1 nM) for 15 min before harvest. The cellular AKT-\( \text{P}^{\text{S}473} \) and AKT were determined by immunoblotting.

Supplementary Figure 9. Effects of chronic Cry1 depletion on gene expression of regulators of insulin signaling and pro-inflammatory genes. (A) The mRNA expression of Socs3 in the liver of Ad-shCry1 vs Ad-shLacZ after 14 days of HFD; (B) The mRNA expression of Socs3 in primary hepatocytes isolated from mice injected with Ad-shCry1 vs Ad-shLacZ after 7 days of HFD; (C) The mRNA expression of pro-inflammatory genes in the liver of mice injected with Ad-shCry1 vs Ad-shLacZ after 14 days of HFD. The data were plotted as mean ± S.D. * \( p \)-value * < 0.05 was determined by paired two-tailed Student’s t-test.
Supplementary Figure 1 (related to Figure 1). (A) Absence of DDB1 in PMHs isolated from Ddb1-LKO mice. (B) Tissue-specific mRNA expression of Ddb1 in WT mice. The data were plotted as mean ± SD (n = 3).
Supplementary Figure 2 (related to Figure 2). High fat diet shows no effects on $Ddb1$ mRNA levels in both the liver and white adipose tissue (WAT). (A) $Ddb1$ mRNA in the liver; (B) $Ddb1$ mRNA in WAT; The data were plotted as mean ± S.D. (n = 5/group).
Supplementary Figure 3 (related to Figure 2). Both $Ddb1^{flox/flox}$ and $Ddb1$-LKO mice were treated with HFD and subjected to GTT and ITT test at indicated times after HFD feeding. The data were plotted as mean ± SEM. (n = 4-5).
**Supplementary Figure 4 (related to Figure 3).** Effects of manipulation of Ddb1 expression on FOXO1 transcriptional activity. (A) 293T cells were transfected with Foxo1 and G6Pase-luc in the presence of either shLacZ or shDdb1. 48 h later, the luciferase activities were measured and normalized by β-gal activity. (B) 293T cells were transfected with expression vectors of Foxo1 and G6Pase-luc in the presence of GFP or Ddb1 plus Cul4a. 48 h later, the luciferase activities were measured and normalized by β-gal activity. The data were plotted as mean ± S.D. (n=3). * p-value * < 0.05 or ** < 0.01 was determined by paired two-tailed Student’s t-test. The experiment was repeated at least 3 times.
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Supplementary Figure 6 (related to Figure 5). Mapping the region of CRY1 protein for its interaction with FOXO1. 293T cells were transfected with vectors encoding Cry1-wt and Cry1 truncation mutants along with Myc-Foxo1. 36 hr later cells were harvested for immunoprecipitation assay with anti-FLAG agarose beads. The presence of Myc-FOXO1 and CRY1-FLAG protein was detected by Western blot with anti-Myc and anti-FLAG.
Supplementary Figure 7 (related to Figure 6). Effects of acute insulin treatment on the Cry1 mRNA in PMHs and the mouse liver. The mRNA was extracted from PMHs treated with 10 nM of insulin for 2 hr or from the mouse liver 2 hr after intra-peritoneal injection with insulin. The Cry1 mRNA level was measured by RT-qPCR analysis. The data were plotted as mean ± S.D. (n= 5-8).
Supplementary Figure 8 (related to Figure 7). Chronic Cry1 depletion reduces hepatocyte response to insulin. (A) Schematic diagram of experiment design; (B) Primary hepatocytes infected with Ad-shCry1 vs Ad-shLacZ were exposed to insulin (1 nM) for 15 min before harvest. The cellular AKT-P^S473 and AKT were determined by immunoblotting.
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