Disruption of the *cereblon* gene enhances hepatic AMPK activity and prevents high fat diet-induced obesity and insulin resistance in mice

**Short running title**
Crbn is a negative modulator of AMPK *in vivo*

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Abstract

A nonsense mutation in cereblon (CRBN) causes a mild type of mental retardation in humans. An earlier study showed that CRBN negatively regulates the functional activity of AMP-activated protein kinase (AMPK) in vitro by binding directly to the $\alpha_1$ subunit of the AMPK complex. However, the in vivo role of CRBN was not studied. To elucidate the physiological functions of Crbn, a mouse strain was generated in which the Crbn gene was deleted throughout the whole body. In Crbn-deficient mice fed a normal diet, AMPK in the liver showed hyper-phosphorylation, which indicated the constitutive activation of AMPK. Since Crbn-deficient mice showed significantly less weight gain when fed a high fat diet and their insulin sensitivity was considerably improved, the functions of Crbn in the liver were primarily investigated. These results provide the first in vivo evidence that Crbn is a negative modulator of AMPK, which suggests that Crbn may be a potential target for metabolic disorders of the liver.
Introduction

Initially, *cereblon* (*CRBN*) was identified as a target gene for a mild type of mental retardation in humans (1) and was subsequently characterized in several different functional contexts. CRBN interacts directly with large-conductance calcium-activated potassium channels and regulates their surface expression (2). Later, CRBN was identified as a primary target for thalidomide-induced teratogenicity and as a substrate receptor for the E3 ligase complex (3). More recently, we reported that CRBN interacts directly with the $\alpha_1$ subunit of AMP-activated protein kinase (AMPK) and inhibits activation of the enzyme *in vitro* (4).

AMPK is a metabolic master switch in response to variations in cellular energy homeostasis (5). The activity of AMPK can be modulated by the phosphorylation of a threonine at position 172 (Thr172) in the $\alpha$ subunit by upstream kinases such as LKB1 (6). AMPK inactivates acetyl-CoA carboxylase (ACC) via direct protein phosphorylation and suppresses expression of lipogenic genes, including fatty acid synthase (FAS), thereby inhibiting fatty acid synthesis (7, 8). AMPK is implicated in the regulation of hepatic glucose and lipid metabolism, thereby affecting the energy status of the whole body (7, 9). Moreover, AMPK was identified as a major pharmacological target protein for the treatment of metabolic diseases. For example, experimental animal models of type-2 diabetes and obesity show that activation of AMPK by metformin or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) reduces blood glucose levels and improves lipid metabolism (10-12).

Our recent study found that CRBN interacted directly with the AMPK $\alpha_1$ subunit both in cultured cell lines and *in vitro*, and the binding sites within the two proteins were localized (4). The levels of the AMPK $\gamma$ subunit and CRBN in the AMPK complex varied in a reciprocal manner, i.e., a higher CRBN content corresponded to lower $\gamma$ subunit content.
AMPK activation was reduced as its γ subunit content was decreased by CRBN. Thus, it was proposed that CRBN may act as a negative regulator of AMPK in vivo (4). The aims of the present study were to test this hypothesis and to understand the physiological role(s) of CRBN by generating Crbn knockout (KO) mice. The results showed that AMPK activity was activated constitutively in Crbn KO mice under normal conditions, and that Crbn KO mice fed a long term HFD showed a marked improvement in their metabolic status.
Research Design and Methods

Generation of Crbn KO mice

To generate Crbn KO mice, heterozygous F1 animals were provided by the knockout mouse service (Macrogen Inc., Seoul, Korea). The targeting vector used to delete a segment containing exon 1 of the Crbn gene (1.1 kb) was constructed using a 5’ short arm fragment (2.6 kb) and a 3’ long arm fragment (7.3 kb), which were ligated into the pOsdupdel vector. The targeting vector was constructed by replacing the 1.1 kb genomic segment with the tk-neomycin cassette. Heterozygous F1 animals were backcrossed with C57BL/6N mice over at least 10 generations before this study. Heterozygous males and females were then bred to produce Crbn KO mice. The genotypes of the WT and Crbn KO mice were determined by RT-PCR using tail genomic DNA and primers specific for WT or Crbn KO alleles (P1, P2, and P3 in Figure 1A).

Experimental animals

Mice were maintained on a standard chow diet and water ad libitum in pathogen-free conditions and housed in a room with a 12 h light-dark cycle. To induce obesity and insulin-resistant phenotypes, male mice (5 weeks old; n = 12–13 per group) were fed a HFD (Research Diet D12492) for 14 weeks while being housed separately. Body weight and food intake were recorded throughout the experiments. Food intake was assessed by determining the difference in food weight during a 7 day period. All experiments were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee.
**Insulin sensitivity**

Glucose tolerance tests were performed by intraperitoneally injecting mice with D-glucose (Sigma) at a dose of 2 g/kg of body weight after a 16 h fast. Insulin tolerance tests were performed intraperitoneally by injecting human insulin (Sigma) at a dose of 0.75 U/kg of body weight after a 4 h fast. Blood samples were collected via the tail vein and plasma glucose levels were measured using a glucometer (Roche Diagnostics).

**Histological analysis of the liver**

At the end of the 14-week period, mice were sacrificed and the livers fixed in 10% formalin and embedded in paraffin. Paraffin sections (5 µm) were then subjected to H&E staining. Cryosections were stained using Oil Red O and counterstained with hematoxylin to visualize the lipid droplets.

**Quantitative real-time PCR analysis**

Total RNA was isolated from liver tissues of the indicated mice with TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. Expression was normalized against the levels of β-actin mRNA. The sequences of the primers used in the PCR analyses are described in Supplementary Table 1.

**Statistical analysis**
All values were expressed as the mean ± SEM. Significant differences between groups were determined using two-tailed unpaired Student’s t-tests and multiple comparisons were performed using one-way ANOVA or two-way repeated-measures ANOVA. Differences with $P < 0.05$ were considered statistically significant and are shown in the Figure legends.
Results

Generation of Crbn KO mice and genotyping

Crbn KO mice were generated to elucidate the in vivo function of Crbn. (Fig. 1A). PCR analysis of genomic DNA from the tails of Crbn KO mice confirmed the loss of the wild type (WT) gene and the presence of the targeting vector (Fig. 1B). CRBN protein was not detected in the liver, skeletal muscle (SKM), or white adipose tissue (WAT) (Fig. 1C), and all other tissues tested by western blot analysis. The daily body weight of both male and female KO mice fed a normal diet from weaning to 12 weeks-of-age was comparable with that of WT mice (Supplementary Fig. 1A).

Our previous study showed that CRBN inhibits the activation of AMPK in vitro by interacting directly with the α1 subunit of AMPK (AMPK α1) (4); therefore, the current study examined whether Crbn deficiency affected AMPK activation (Fig. 1D). First, the phosphorylation of AMPK Thr172 was measured in primary mouse embryonic fibroblasts (MEFs). The amount of phosphorylated AMPK α (P-AMPK α) increased in Crbn +/- and Crbn -/- MEFs (Fig. 1E). ACC is inactivated by phosphorylation of serine79 after AMPK activation; therefore, we next measured the levels of phosphorylated ACC (P-ACC). Increases in P-AMPK were accompanied by higher levels of P-ACC in Crbn +/- and Crbn -/- MEFs compared with Crbn +/- MEFs (Fig. 1F). It was shown previously that the binding of exogenous CRBN to AMPK decreases the amount of γ subunits in the AMPK complex (4); therefore the effects of Crbn KO on the AMPK complex were tested by immunoprecipitating the endogenous AMPK complex from MEFs (Supplementary Figs. 1C–F). The intensity of the AMPK β band did not change greatly (Supplementary Fig. 1D); however, the intensity of the γ1 subunit band was significantly higher in both Crbn +/- and Crbn -/- MEFs compared
with Crbn +/+ (Supplementary Fig. 1E). These results support the hypothesis that CRBN suppresses AMPK activation by reducing the affinity of the γ1 subunit for the AMPK complex (4).

**AMPK is activated in Crbn KO mice**

To test whether Crbn deficiency affected the function of AMPK in vivo, the enzymatic activity of AMPK was assessed in the mouse liver by determining its phosphorylation state. AMPK phosphorylation was 8.2-fold higher in Crbn KO mice than in WT (Fig. 2A and Supplementary Fig. 2A). Subsequently, the effects of Crbn KO were investigated under conditions known to activate endogenous AMPK (13, 14). The level of AMPK phosphorylation increased in a time-dependent manner in both WT and Crbn KO mice after injection of metformin; however, the level of P-AMPK α was higher in Crbn KO mice than that in WT (Fig. 2B and Supplementary Fig. 2B). Similar results were obtained with primary MEFs cultured under serum-deprived conditions. (Supplementary Fig. 2C and D). Overall, these results suggest that AMPK is hyperactivated in the absence of Crbn in vivo, which further indicates that Crbn acts as an endogenous negative regulator of AMPK.

**Crbn deficiency has a protective effect against HFD-induced obesity**

To elucidate the physiological role(s) of Crbn in vivo, both WT and Crbn KO mice were fed either a normal chow diet or a HFD (Fig. 3A and B). The HFD-induced weight increase was slower and the level of weight gain was much less for Crbn KO mice than for the WT. The body weight of WT mice fed the HFD was significantly higher than that of mice fed the chow
diet at 2 weeks, whereas the Crbn KO mice were significantly heavier at 7 weeks (Fig. 3E). The difference in the HFD-induced weight gain of WT and Crbn KO mice was not attributable to their food consumption (Fig. 3F). The initial average weight of Crbn KO mice was also not significantly different from that of WT mice (Fig. 3E and Supplementary Table 2).

Necropsies were performed on both WT and Crbn KO mice, which showed that Crbn KO mice fed the HFD had a lower epididymal fat mass than WT (Fig. 3C and D). After 14 weeks on the HFD, the average epididymal fat mass of WT mice was 2.7-fold higher than that of Crbn KO. Crbn KO mice fed with a normal chow diet had lower epididymal fat masses than WT (Fig. 3G). These observations suggest that mice lacking Crbn experienced greater protection from body fat accumulation and obesity caused by a high fat intake.

_Crbn KO mice are resistant to diet-induced fatty liver_

The effects of HFD on the morphology and lipid content of the liver in Crbn KO mice were tested next. A comparison of the livers from HFD-fed WT and Crbn KO mice showed that WT livers were much larger, heavier, and paler than those of Crbn KO mice (Fig. 3D and Fig. 4A). The hepatic triglyceride (TG) content was significantly lower in HFD-fed Crbn KO mice than in the WT. The TG content was also lower in Crbn KO mice fed the normal chow diet than in WT (Fig. 4B). Hepatic cholesterol levels were also higher in both WT and Crbn KO mice fed a HFD (Fig. 4C).

In agreement with these results, hematoxylin and eosin (H&E) staining of liver sections showed that the livers of HFD-fed WT mice contained unstained lipid inclusions, which were
less abundant in the livers of HFD-fed Crbn KO mice (Figs. 4D–G). Oil Red O staining of lipids further confirmed the massive accumulation of neutral lipids in the livers of WT mice fed a HFD, which is a hallmark of fatty liver, but not in the livers of Crbn KO fed a HFD (Figs. 4H–K). Thus, Crbn deficiency prevented the accumulation of fats in the epididymal tissues and liver, which made the livers more resistant to fatty liver, which is normally caused by high fat intake.

_Crbn KO mice fed a HFD show improved glucose homeostasis and insulin sensitivity_

Crbn KO mice were largely protected from HFD-induced obesity and fatty liver; therefore we next investigated their metabolic parameters. When fed a HFD, WT mice showed significantly higher levels of serum glucose, insulin, and leptin than mice fed normal chow (Fig. 5A–C), which suggests impaired insulin sensitivity. However, the serum levels of glucose, insulin, and leptin were significantly lower in HFD-fed Crbn KO mice. Other plasma metabolic parameters were also measured in both WT and Crbn KO mice fed normal chow or the HFD (Supplementary Fig. 3). Under both diet conditions WT and Crbn KO mice showed similar serum levels of TG, cholesterol, resistin, adiponectin, TNF-α, MCP-1 and PAI-1. By contrast, the serum non-esterified free fatty acid (FFA) levels of Crbn KO mice fed a HFD were lower than those in the WT (Supplementary Fig. 3C), which agrees with a previous report showing that type-2 diabetic patients with a fatty liver are substantially more insulin-resistant and have higher levels of plasma FFA (15). Increased glucose tolerance and insulin sensitivity were also confirmed in HFD-fed Crbn KO mice. (Fig. 5D and E). These results demonstrate that Crbn deficiency may prevent glucose intolerance and insulin resistance, which are normally induced by a long term HFD.
Crbn deficiency alters lipid metabolism and glucose metabolism in the liver

To elucidate the molecular basis of the phenotypic changes observed in Crbn KO mice fed a HFD, expression profiling was performed for several metabolic enzymes. AMPK activation was also monitored. The level of P-AMPK was lower in mice fed a HFD (Fig. 6A), which is in agreement with previous studies (16, 17). The total AMPK expression level was not different between WT and Crbn KO mice fed a normal chow diet or a HFD, whereas P-AMPK was significantly higher in Crbn KO mice fed a HFD than in WT mice fed a HFD (Fig. 6B). Furthermore, the ratio of P-ACC to total ACC was consistent with the level of AMPK α activation (Fig. 6D). Despite the hyper-phosphorylation of AMPK, the expression levels of the AMPK upstream kinases, LKB1 and CaMKKβ, were similar in Crbn KO mice and WT mice (Supplementary Fig. 4), suggesting that these kinases may not be involved in AMPK activation in Crbn KO mice.

There were no statistical differences in the expression of SREBP1C and ChREBP (Fig. 7A and B), a major regulator of lipogenesis (18, 19) between the groups; however, the expression of PPARγ, a lipogenic transcription factor (20, 21), was significantly lower in Crbn KO fed either a normal chow diet or a HFD compared with that in WT mice fed these diets (Fig. 7C). The expression of FAS, which is involved in fatty acid synthesis, and DGAT2, a rate-limiting enzyme that catalyzes the final step of TG synthesis (22, 23), was reduced in Crbn KO mice fed a normal chow diet or the HFD (Fig. 7D and E). These results are consistent with the finding that the level of hepatic TG was lower in Crbn KO mice fed both the normal chow diet and the HFD compared with that in the WT mice (Fig. 4B). The expression levels of ACC1 and SCD1 mRNA were significantly lower in HFD-fed Crbn KO mice than in HFD-fed WT (Fig. 7F and G). Expression of G6Pase, but not PEPCK, was significantly lower in
Crbn KO mice fed both the normal chow diet and the HFD than in WT (Fig. 7H and I). The expression of _L-PK_, which is a key enzyme involved in glycolysis, was significantly higher in WT mice fed the HFD; however, its upregulation was completely abrogated in Crbn KO mice fed the HFD (Fig. 6L). This result is consistent with serum glucose levels (Fig. 5A), because _L-PK_ gene transcription is positively-regulated by glucose and insulin (24, 25). The pattern of AMPK activation (Fig. 6A) is also consistent with the reciprocal pattern of mRNA expression for _L-PK_ and _G6Pase_ (Fig. 7M and I); AMPK activation inhibits the expression of _L-PK_ (25, 26) and _G6Pase_ (27). Expression of _FGF21_ was 10.3-fold higher in WT mice fed a HFD than in WT fed a normal chow diet (Fig. 6J), which is consistent with a previous report showing that obesity may be considered an FGF21-resistant state (28). However, _FGF21_ expression was only 3.7-fold higher in Crbn KO mice fed a HFD than that in Crbn KO mice fed normal chow. The mRNA expression levels of _HMGCS_, which is involved in cholesterol biosynthesis were 1.8-fold higher in HFD-fed WT mice compared with those in normal chow-fed WT mice (Fig. 7K). Unexpectedly, _HMGCS_ expression was 1.7-fold higher in Crbn KO mice fed normal chow than in WT fed normal chow. The induction of _HMGCS_ in Crbn KO mice fed a normal chow diet did not correlate with the observed phenotypes of the WT and Crbn KO mice. This may be due to compensatory gene induction to maintain hepatic homeostasis. There were no changes in the expression of _Dher24_, which is involved in cholesterol biosynthesis and _HSL_, which is involved in lipolysis (Fig. 7L and N). The protein expression patterns of ACC1, FAS, SCD1, G6Pase, and Crbn (Fig. 6A, C, and E–H) consistent with the mRNA expression profiles shown in Fig. 7. Expression of ACC1 was also increased in WT mice fed a HFD compared with those fed a normal chow diet (Fig. 6E). Overall, these results show that the disruption of Crbn affects the expression of many key metabolic genes. The expression of several lipogenic and gluconeogenic proteins, which are upregulated by HFD,
was significantly lower in the livers of Crbn KO mice, which may be explained by the constitutive activation of AMPK (23, 25-27, 29).
Discussion

Crbn is evolutionarily conserved between plants and animals, and is expressed widely in various mammalian tissues. To validate the in vivo function of Crbn as a novel regulator of AMPK, Crbn KO mice were established. The Crbn KO mice were viable, with no apparent defects in gross morphology or basic behavior.

As suggested by a previous knockdown study of endogenous CRBN in cultured cell lines (4), endogenous AMPK was constitutively hyperactivated in Crbn KO mice under normal conditions in all tissues tested; therefore, Crbn KO mice were fed a HFD before assessing the effects of Crbn deficiency on several parameters: body weight, fatty liver, glucose homeostasis, insulin resistance, and metabolic parameters. In general, Crbn KO mice fed a HFD showed a significant improvement in their metabolic profiles compared with HFD-fed WT control mice. Also, Crbn KO mice showed no signs of metabolic syndrome, even after 14 weeks on a HFD. Fat accumulation within the epididymal tissue and liver were also markedly reduced in KO mice. Thus, it was hypothesized that hyperactivity of AMPK in Crbn KO mice was the major contributor to the overall improvement in lipid and glucose homeostasis and insulin sensitivity. It was intriguing that Crbn expression was significantly upregulated, whereas that of P-AMPK was correspondingly downregulated, in the livers of WT mice on a HFD. This observation agreed with our previous prediction that Crbn might negatively regulate the functional activity of AMPK in vivo (4).

The current study focused on the effects of Crbn KO on the liver and hepatic metabolism because the liver plays a key role in controlling overall energy status, whereas AMPK coordinates changes in the hepatic enzymes involved in carbohydrate and lipid metabolism. Activation of hepatic AMPK inhibits lipogenesis, cholesterol synthesis, and glucose
production (7, 8, 30). The lower expression of lipogenic regulators, including FAS, ACC1, SCD1, PPARγ, and DGAT2, observed in HFD-fed Crbn KO mice suggests that inhibition of hepatic lipogenesis may contribute to lower levels of fat accumulation. The hepatic expression of G6Pase was lower in Crbn KO mice fed a HFD, suggesting lower levels of gluconeogenesis. Consistent with these findings, the glucose and insulin tolerance tests showed restoration of normal levels in Crbn-deficient mice fed a HFD. Infection with Ad-DN-AMPK increased the glucose output of primary Crbn KO hepatocytes in a dose-dependent manner in comparison to Crbn KO hepatocytes infected with Ad-GFP (Supplementary Fig. 5). Collectively, this suggests that the primary mechanism by which KO of Crbn reduces lipogenesis and gluconeogenesis in the liver operates, at least in part, by regulating AMPK and ACC activity via protein phosphorylation. Furthermore, the levels of Crbn protein and P-AMPK showed a strongly correlation in vivo, because the levels of Crbn expression increased as P-AMPK expression decreased in WT mice fed a HFD.

There were several noticeable changes in the expression of key metabolic genes in Crbn KO mice fed a regular chow diet, suggesting a physiological role for Crbn under normal conditions. For example, the expression of lipogenic genes, such as FAS, PPARγ, and DGAT2, and a gluconeogenic gene, G6Pase, was significantly lower. In a good agreement with these findings, hepatic TG levels and the epididymal fat mass were lower in Crbn KO mice fed a regular diet than in WT fed a regular diet. However, morphological phenotypes, including body weight, liver weight, liver morphology, liver section, glucose tolerance, and insulin sensitivity were similar in WT and Crbn KO mice before the HFD was started. Therefore, Crbn deficiency conferred greater resistance to a metabolic syndrome phenotype under severe pathophysiological conditions (such as a HFD), but not under normal physiological conditions.
In our previous report, AMPK α₁ was identified as a CRBN-binding protein (4). Two isoforms of the AMPK α subunit, AMPK α₁ and AMPK α₂, are found in mammals (31). AMPK complexes containing each α subunit isoform were equally represented in terms of total AMPK activity in the liver (26). The current study examined the interaction between AMPK α₂ and Crbn. There was no difference in the binding affinity of Crbn for AMPK α₁ or AMPK α₂ (Supplementary Fig. 6). This was because the putative Crbn-binding site in AMPK α₁ is within a region covering amino acids 394–422 (4), which is also highly conserved in AMPK α₂, (within a region covering amino acids 388–417). These results suggested that Crbn can modulate cellular AMPK, irrespective of its subtype.

It is important to determine whether the Crbn-dependent inhibition of AMPK is also conserved in other organisms, especially humans. Our previous study showed that the activity of AMPK was inhibited by the expression of exogenous Crbn in human, rat, and mouse cell lines (4), so it is likely that the negative regulation of AMPK by CRBN also occurs in humans. Recently, CRBN, which is located in the 3p26–25 region in humans, was identified as a target gene for obesity and insulin. Several single nucleotide polymorphisms (SNPs) close to the CRBN gene are associated with central obesity and high blood pressure in humans and the mice (32), indicating the potential clinical relevance of CRBN in metabolic syndromes.

However, several important questions still need to be addressed. First, the regulation of Crbn gene expression is not understood. Crbn expression was upregulated in the long term by a high fat intake (Fig. 6O and 7C). This observation implies that Crbn is induced by a HFD and that CRBN regulates AMPK activity via a negative-feedback loop in vivo. Interestingly, at least three putative sterol regulatory elements (SRE) and one putative PPARγ binding site are located within -850 bp upstream of the promoter region within the mouse Crbn gene. These
two transcriptional factors regulate lipogenesis, which may provide mechanistic insights into the nutrient-dependent modulation of Crbn expression. Second, it is not clear how AMPK is regulated by Crbn in other organs. The physiological roles of AMPK are established in other organs such as adipose tissue and SKM, but it was not possible to discern the contributions of these organs to the metabolic phenotype in Crbn KO mice. The activation of AMPK in WAT and SKM was higher in Crbn KO mice than in WT mice (Supplementary Fig. 7). Thus, it is feasible that other organs relative to metabolism may be affected in a similar way to the liver in Crbn KO mice.

The liver is regarded as the core center for maintaining glucose homeostasis and lipid metabolism, so understanding the normal physiology and the pathophysiology of hepatic metabolism is a prerequisite to understanding whole-body metabolism (7, 9, 26). Of a variety of tissues tested, the activity of AMPK is lowest in brain and SKM, and highest in the liver (33). This may underlie why deletion of Crbn, a negative regulator of AMPK, most affects the liver in Crbn KO mice. In addition, alterations in liver function clearly affect whole-body metabolism and underlie the development of metabolic diseases, including type-2 diabetes and metabolic syndromes (7, 9). Thus, the current study of the role of Crbn in hepatic metabolism may be the first step toward a greater understanding of the physiological function of Crbn at the molecular level during normal and diseased states.

In summary, this study provides the first in vivo evidence that Crbn negatively regulates the activation of AMPK and that Crbn deficiency protects mice from obesity, fatty liver, and insulin resistance caused by a HFD. Thus, CRBN may be considered as a novel regulator of body metabolism and energy homeostasis.
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Figure Legends

Figure 1. Generation of Crbn KO mice.

(A) The vector construct used to generate Crbn KO (Crbn -/-) mice. The genotyping primers are indicated as P1, P2, and P3.

(B) Genotypes of the WT (Crbn +/+) heterozygous KO (Crbn +/-), and homozygous KO (Crbn -/-) mice were determined by RT-PCR using tail genomic DNA.

(C) Protein extracts from liver, SKM, or WAT of Crbn +/+ and Crbn -/- mice showing the levels of endogenous Crbn protein as determined by western blotting. β-actin was used as the loading control. (n = 4 per group).

(D) Western blots of endogenous AMPK α, P-AMPK α, ACC, and P-ACC in Crbn +/+, Crbn +/-, and Crbn -/- primary MEFs. GAPDH was used as the loading control. The results shown are representative of four independent experiments.

(E and F) Relative band intensities as determined by densitometric analysis of the blots in (D). Error bars represent the SEM.

Figure 2. Increased AMPK activation in the Crbn -/- liver.

(A) Proteins extracted from the livers of Crbn +/+ and Crbn -/- mice were separated by SDS-PAGE and immunoblotted with anti-AMPK α, anti-P-AMPK α, and anti-β-actin antibodies. Nine-week-old male mice were used (n = 9 per group). β-actin was used to confirm equal protein loading. Error bars represent the SEM.
(B) Liver lysates were prepared and subjected western blot analysis with anti-AMPK α, anti-P-AMPK α, and anti-β-actin antibodies. The numbers represent the time after intraperitoneal injection of metformin at a dose of 150 mg/kg of body weight. Nine-week-old male mice were used (n = 5 per group). β-actin was used to confirm equal protein loading. Error bars represent the SEM.

**Figure 3. Crbn deficiency prevented HFD-induced obesity.**

(A–D) Representative images of mice fed a normal chow diet or a HFD at the end of the 14 week experimental period.

(E) Body weight changes in WT and Crbn knockout mice fed a normal chow diet or a HFD were monitored weekly during the 14 week experimental period.

(F) Cumulative food consumption of WT and Crbn KO mice fed a HFD for 14 weeks.

(G) Epididymal fat mass of mice at the end of the 14 week experiment.

Error bars represent the SEM (n = 12–13 per group).

*Statistical differences (*P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001) versus WT mice fed the same diet.

†Statistical differences (†P < 0.05, ††P < 0.01, †††P < 0.005, ††††P < 0.001) versus mice with the same genotype that were fed the normal chow diet.
Figure 4. Crbn deficiency in the mouse liver prevented fatty liver.

(A) Liver mass of WT and Crbn KO mice fed a normal chow diet or HFD at the end of the 14 week experimental period.

(B) Hepatic triglyceride (TG) levels.

(C) Hepatic cholesterol levels.

(D–G) Liver sections of the indicated mice were stained with H&E. Scale bar = 50 µm.

(H–K) Lipids in the liver section of the indicated mice were stained with Oil Red O. Scale bar = 50 µm.

Error bars represent the SEM (n = 9–10 per group).

*Statistical differences (*P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001) versus WT mice fed the same diet.

†Statistical differences (†P < 0.05, ††P < 0.01, †††P < 0.005, ††††P < 0.001) versus mice with the same genotype that were fed the normal chow diet.

Figure 5. Glucose homeostasis and insulin sensitivity in Crbn -/- mice under high fat diet conditions.

(A) Plasma glucose concentration, (B) plasma insulin concentration, and (C) plasma leptin concentration in WT and Crbn KO mice fed a normal chow diet or a HFD at the end of the 14 week experimental period. (D) Intraperitoneal glucose tolerance test (GTT). (F) Intraperitoneal insulin tolerance test (ITT).
Error bars represent the SEM (n = 9–10 per group).

*Statistical differences (\(^*\)P < 0.05, \(\text{**}P < 0.01, \text{***}P < 0.005, \text{****}P < 0.001\)) versus WT mice fed the same diet.

†Statistical differences (\(\text{†}P < 0.05, \text{††}P < 0.01, \text{†††}P < 0.005, \text{††††}P < 0.001\)) versus mice with the same genotype that were fed the normal chow diet.

Figure 6. **Crbn KO mice fed a HFD showed AMPK activation and ACC inhibition in the liver.**

(A) Western blotting analysis of AMPK \(\alpha\), P-AMPK \(\alpha\), Crbn, ACC, P-ACC, ACC1, FAS, SCD1, and G6Pase proteins levels in liver tissue lysates. \(\beta\)-actin was used as the loading control. Asterisks denote nonspecific bands.

(B) The ratio of phosphorylated AMPK \(\alpha\) to AMPK \(\alpha\). (C) The ratio of Crbn to \(\beta\)-actin. (D) The ratio of phosphorylated ACC to total ACC. (E) The ratio of ACC1 to \(\beta\)-actin. (F) The ratio of FAS to \(\beta\)-actin. (G) The ratio of SCD1 to \(\beta\)-actin. (H) The ratio of G6Pase to \(\beta\)-actin on the blot in (A). Error bars represent the SEM (n = 9–10 per group). *Statistical differences (\(^*\)P < 0.05, \(\text{**}P < 0.01, \text{***}P < 0.005, \text{****}P < 0.001\)) versus WT mice fed the same diet.

†Statistical differences (\(\text{†}P < 0.05, \text{††}P < 0.01, \text{†††}P < 0.005, \text{††††}P < 0.001\)) versus mice with the same genotype that were fed the normal chow diet.

Figure 7. Deletion of Crbn resulted in the defective expression of genes involved in hepatic glucose and lipid metabolism.
(A–O) Total RNA was isolated from the liver tissues of the indicated mice and subjected to quantitative real-time PCR analysis to determine the expression of: (A) sterol regulatory element-binding protein-1c (SREBP1C), (B) carbohydrate-responsive element-binding protein (ChREBP), (C) peroxisome proliferator-activated receptor gamma (PPARγ), (D) fatty acid synthase (FAS), (E) diacylglycerolacyltransferase 2 (DGAT2), (F) acetyl-CoA carboxylase 1 (ACC1), (G) stearoyl-CoA desaturase 1 (SCD1), (H) phosphoenolpyruvate carboxykinase (PEPCK), (I) glucose-6-phosphatase (G6Pase), (J) fibroblast growth factor 21 (FGF21), (K) 3′-hydroxymethyl glutaryl coenzyme A synthase (HMGCS), (L) 24-dehydrocholesterol reductase (Dhcr24), (M) liver-type pyruvate kinase (L-PK), (N) hormone-sensitive lipase (HSL), and (O) Crbn. Expression was normalized against β-actin mRNA levels. Fold changes in the mRNA levels relative to WT mice fed a normal chow diet, which were set arbitrarily at 1.0, are shown. Error bars represent the SEM (n = 9–10 per group).

*Statistical differences (*P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001) versus WT mice fed the same diet.

†Statistical differences (†P < 0.05, ††P < 0.01, †††P < 0.005, ††††P < 0.001) versus mice with the same genotype that were fed the normal chow diet.
Figure 1. Generation of Crbn KO mice.
152x181mm (300 x 300 DPI)
Figure 2. Increased AMPK activation in the Crbn -/- liver.

102x62mm (300 x 300 DPI)
Figure 3. Crbn deficiency prevented HFD-induced obesity.
205x396mm (300 x 300 DPI)
Figure 4. Crbn deficiency in the mouse liver prevented fatty liver.
Figure 5. Glucose homeostasis and insulin sensitivity in Crbn -/- mice under high fat diet conditions.

99x71mm (300 x 300 DPI)
Figure 6. Crbn KO mice fed a HFD showed AMPK activation and ACC inhibition in the liver.
Figure 7. Deletion of Crbn resulted in the defective expression of genes involved in hepatic glucose and lipid metabolism.

128x92mm (300 x 300 DPI)
SUPPLEMENTARY DATA

Figure Legends

Supplementary Figure 1. Characterization of Crbn -/- mice under normal conditions.

(A) Body weight changes in WT and Crbn KO mice of each gender were monitored daily from P21 to P84 (n = 30–35 per group).

(B) Primary MEF (Crbn +/+; WT; Crbn+/–: Het; or Crbn–/–: Homo) lysates were solubilized using RIPA buffer and immunoprecipitated with either mouse IgG or mouse anti-AMPK α. The precipitates were separated by SDS-PAGE and immunoblotted with anti-AMPK α, anti-AMPK β, anti-AMPK γ1, and anti-Crbn antibodies.

(C–E) Relative band intensities as determined by densitometric analysis of the blot shown in (B). The results are representative of four independent experiments. Error bars represent the SEM.

Supplementary Figure 2. Crbn deficiency increased AMPK activity under conditions known to activate endogenous AMPK.

(A) Long exposure films of the middle blot shown in Fig. 2A.

(B) Long exposure films of the middle blot shown in Fig. 2B.

(C) Primary MEFs (Crbn +/+; WT; Crbn+/–: Het; or Crbn–/–: Homo) were incubated for the indicated times (10, 30, and 60 min) in nutrient-free medium (Hank’s Buffered Salt Solution, HBSS) after the removal of serum for 3 h. Lysates were immunoblotted with anti-AMPK
α and anti-P-AMPK α antibodies. β-actin was used to confirm equal protein loading. The results shown are representative of four independent experiments.

(D) Relative band intensities as determined by densitometric analysis of the blot shown in (C). Error bars represent the SEM.

Supplementary Figure 3. Analysis of plasma metabolic parameters.

(A) Plasma levels of TG, (B) cholesterol, (C) FFA, (D) resistin, (E) adiponectin, (F) tumor necrosis factor-α (TNF α), (G) monocyte chemotactic protein-1 (MCP-1), and (H) plasminogen activator inhibitor-1 (PAI-1) in the indicated mice. Error bars represent the SEM (n = 9–10 per group).

*Statistical differences (\(^{*} P < 0.05, \,** P < 0.01, \, *** P < 0.005, \, **** P < 0.001\)) versus WT mice fed the same diet. †Statistical differences (\(^{†} P < 0.05, \, †† P < 0.01, \, ††† P < 0.005, \, †††† P < 0.001\)) versus mice with the same genotype that were fed the normal chow diet.

Supplementary Figure 4. The hepatic expression of AMPK upstream kinases was not altered in Crbn KO mice.

(A) Western blotting analysis of LKB1 and CaMKKβ proteins levels in liver tissue lysates. β-actin was used as the loading control.

(B) The ratio of LKB1 to β-actin. (C) The ratio of CaMKKβ to β-actin on the blot in (A). Error bars represent the SEM (n = 9–10 per group). *Statistical differences (\(^{*} P < 0.05, \,** P < 0.01, \, *** P < 0.005, \, **** P < 0.001\)) versus WT mice fed the same diet.
†Statistical differences (†P < 0.05, ††P < 0.01, †††P < 0.005, ††††P < 0.001) versus mice with the same genotype that were fed the normal chow diet.

Supplementary Figure 5. Overexpression of dominant-negative AMPK mutant abrogated the activation of AMPK following Crbn deletion

Primary hepatocytes were isolated from WT and Crbn KO mice. The hepatocytes were infected with an adenovirus containing GFP (Ad-GFP) or dominant-negative AMPK mutant (Ad-DN-AMPK) at a multiplicity of infection (MOI) of 10 or 30. The glucose output assay was performed 24 h after adenoviral infection.

The results shown are representative of four independent experiments. Error bars represent the SEM. ***P < 0.005 versus WT hepatocytes that were infected with Ad-GFP. †††P < 0.005 versus Crbn KO hepatocytes that were infected with Ad-GFP.

Supplementary Figure 6. Crbn interacted with both AMPK α1 and AMPK α2.

HEK293FT cells were transiently co-transfected with either HA::Crbn or an HA empty vector, or with Myc::AMPK α1, Myc::AMPK α2 or a Myc empty vector. Cells were harvested after 24 h and Crbn was immunoprecipitated using an anti-HA antibody. Western blots of the immunoprecipitates were probed with either anti-Myc or anti-HA antibodies. The plus and minus symbols indicate the presence or absence of each protein. The results shown are representative of four independent experiments.
Supplementary Figure 7. AMPK was activated in SKM and WAT of Crbn KO mice fed a HFD.

(A) Western blotting analysis of AMPK α, P-AMPK α, and Crbn proteins levels in SKM lysates. β-actin was used as the loading control.

(B) The ratio of phosphorylated AMPK α to AMPK α. (C) The ratio of Crbn to β-actin on the blot in (A).

(D) Western blotting analysis of AMPK α, P-AMPK α, and Crbn proteins levels in WAT lysates. β-actin was used as the loading control.

(E) The ratio of phosphorylated AMPK α to AMPK α. (F) The ratio of Crbn to β-actin on the blot in (D). *Statistical differences (\(^{*}P < 0.05, \^{**}P < 0.01, \^{***}P < 0.005, \^{****}P < 0.001\)) versus WT mice fed the same diet.

†Statistical differences (\(^{†}P < 0.05, \^{††}P < 0.01, \^{†††}P < 0.005, \^{††††}P < 0.001\)) versus mice with the same genotype that were fed the normal chow diet.
Supplementary Figure 1. Characterization of Crbn -/- mice under normal conditions.
Supplementary Figure 2. Crbn deficiency increased AMPK activity under conditions known to activate endogenous AMPK.

191x391mm (300 x 300 DPI)
Supplementary Figure 3. Analysis of plasma metabolic parameters.
Supplementary Figure 4. The hepatic expression of AMPK upstream kinases was not altered in Crbn KO mice.
Supplementary Figure 5. Overexpression of dominant-negative AMPK mutant abrogated the activation of AMPK following Crbn deletion.
Supplementary Figure 6. Crbn interacted with both AMPK α1 and AMPK α2.

|         | Total | IP : Anti-HA |
|---------|-------|--------------|
| myc     | +     | -            |
| myc::AMPK α1 | -     | +            |
| myc::AMPK α2 | -     | +            |
| HA      | +     | +            |
| HA::CRBN | -     | -            |

Supplementary Figure 6. Crbn interacted with both AMPK α1 and AMPK α2.

55x34mm (300 x 300 DPI)
Supplementary Figure 7. AMPK was activated in SKM and WAT of Crbn KO mice fed a HFD.
126x91mm (300 x 300 DPI)
Supplementary Table 1. Primer sequences used for the PCR analysis

| Gene       | Forward primer            | Reverse primer            |
|------------|---------------------------|---------------------------|
| CRBN<sup>1</sup> | AGACCAGCGAACCTCCCTTA (P1) | GGTGGCAGATCAGTACACCCG (P2) |
| CRBN<sup>2</sup> | AGACCAGCGAACCTCCCTTA (P1) | GCCGTCAGATCAGTACACCCG (P3) |
| CRBN<sup>3</sup> | TCGCTGGGTGATTTCCATT | AGCCGTTGATATGCCTCCG |
| CRBN       | AGCATGGTGCGGAACCTTAAC    | ATCTCTGCTGTGGTCCCAAC     |
| GAPDH      | TCACTGCCACCCAGAAGAC      | TGGCCTCATGAGTAATACCCT    |
| β-actin    | GAAATCTGGGCTGAGGACCAAA  | TGGCCTCATGAGTAATACCCT    |
| SREBP1C    | TGGGCTGGTACCCCAATGC      | TGGCCTCATGAGTAATACCCT    |
| ChREBP     | CCAGCTCAAGGAGGCAAAA      | CATGCTCTCTGAGGAGGAGGAC   |
| FAS        | GGGGCTGGTAGGATGCGGTAAT   | GGGTGAATCCATAGAGGGCACAG  |
| ACC1       | ATGGGGCAGGGCAAGCTTA     | CCGCTCTCTGAGGAGGAGGAC   |
| SCD1       | AGATCTGAGGTCTTACACGACCAC| GACGGATGCTCTCTTCCAGGAC   |
| PPARγ      | AAGGCGAGGCGAGCCTTTG     | ATCTCAAGGAGCTGAGGAGGAC   |
| DGAT2      | TTCTCTGGGACAAGGCCCTATT  | ATCTCAAGGAGCTGAGGAGGAC   |
| PEPCK      | CTCAGCTGATAACGGGCTCTTT  | CTTCAGCTTGAGGAGGACAC     |
| G6Pase     | TCTGTCGGCGATCTACCTGG    | GTAGAATCCAAGGCCGAGAAC    |
| FGF21      | GTGTCAAGGCTCTGTACGCTT   | GGTACAATGCTGTACGGCTC     |
| HMGCS      | GCCGTGAACTGGTGCGCAA     | GCATATAGCAATGTCTCTGCAA   |
| Dhcr24     | GCCGAGCAGCAGCTCTGGTTCC  | TGGCAGCAAGCAGATGGGTG     |
| L-PK       | CTGGAACACCTGCTGCTTCTG   | CACAAATCCACCTCCGACTC     |
| HSL        | CGAGATGTCACAGCATAGGA    | CAGAGGCCGAAGAAAAG        |

<sup>1</sup>Genotyping of WT alleles. <sup>2</sup>Genotyping of targeted allele. <sup>3</sup>Primer sets used in the RT-PCR experiments. All other undesignated primers were used in the real-time PCR experiments. All primers were designed for <i>Mus musculus</i>. 
Supplementary Table 2. Phenotypic parameters and mRNA levels in mice used in this study

| Parameters                        | WT-Chow   | CRBN KO-Chow | WT-HFD   | CRBN KO-HFD |
|----------------------------------|-----------|--------------|----------|-------------|
| Number of mice                   | 12        | 12           | 12       | 13          |
| Initial body weight (g)          | 15.01 ± 0.16 | 15.09 ± 0.45 | 15.31 ± 0.42 | 15.26 ± 0.59 |
| Final body weight (g)            | 26.05 ± 0.25 | 26.79 ± 0.65 | 43.01 ± 1.03 | 33.69 ± 1.93 |
| Gained body weight (g)           | 11.04 ± 0.16 | 11.7 ± 0.36  | 27.7 ± 0.84 | 18.43 ± 1.96 |
| Epididymal fat mass (g)          | 0.6 ± 0.03 | 0.32 ± 0.02  | 2.74 ± 0.09 | 1.02 ± 0.36 |
| Liver mass (g)                   | 0.98 ± 0.03 | 1.09 ± 0.04  | 1.38 ± 0.08 | 1.14 ± 0.05 |
| **Metabolic Profile**            |           |              |          |             |
| Hepatic triglycerides (mg/g)     | 99.32 ± 7.43 | 56.97 ± 6.99 | 143.65 ± 9.13 | 91.02 ± 12.77 |
| Hepatic cholesterol (mg/g)       | 17.51 ± 0.60 | 16.65 ± 0.66 | 24.88 ± 0.97 | 22.84 ± 0.99 |
| Plasma glucose (mg/dl)           | 71.71 ± 3.01 | 74.00 ± 5.16 | 118.14 ± 6.2 | 82.75 ± 3.54 |
| Plasma insulin (ng/ml)           | 0.49 ± 0.08 | 1.19 ± 0.32  | 7.95 ± 1.23 | 1.9 ± 0.6 |
| Plasma leptin (ng/ml)            | 2.03 ± 0.66 | 1.04 ± 0.38  | 38.26 ± 4.81 | 8.93 ± 3.77 |
| Plasma triglycerides (mg/dl)     | 45.59 ± 4.95 | 63.14 ± 6.44 | 54.68 ± 3   | 46.63 ± 6.6 |
| Plasma cholesterol (mg/dl)       | 58.98 ± 1.81 | 65.28 ± 1.54 | 134.35 ± 8.66 | 124.23 ± 12.52 |
| Plasma free fatty acids (mmol/l) | 0.34 ± 0.07 | 0.43 ± 0.06  | 0.37 ± 0.03 | 0.26 ± 0.04 |
| Plasma resistin (ng/ml)          | 0.83 ± 0.08 | 1.06 ± 0.07  | 2.73 ± 0.22 | 2.02 ± 0.42 |
| Plasma adiponectin (µg/ml)       | 10.71 ± 1.03 | 11.07 ± 1.05 | 12.85 ± 0.55 | 11.82 ± 0.92 |
| Plasma TNF-α (pg/ml)             | 9.12 ± 0.45 | 9.39 ± 0.18  | 8.66 ± 0.18 | 9.19 ± 0.16 |
| Plasma MCP-1 (pg/ml)             | 30.95 ± 0.88 | 33.72 ± 1.49 | 29.59 ± 0.61 | 30.16 ± 1.2  |
| Plasma PAI-1 (ng/ml)             | 0.36 ± 0.07 | 0.32 ± 0.05  | 0.52 ± 0.13 | 0.58 ± 0.09 |
| **Liver mRNAs**                  |           |              |          |             |
| SREBP1C                           | 1.00 ± 0.08 | 0.88 ± 0.10  | 1.26 ± 0.09 | 1.06 ± 0.19 |
| ChREBP                           | 1.00 ± 0.06 | 0.83 ± 0.05  | 0.92 ± 0.05 | 0.89 ± 0.05 |
| PPARγ                            | 1.00 ± 0.07 | 0.47 ± 0.07  | 1.12 ± 0.07 | 0.56 ± 0.05 |
| FAS                              | 1.00 ± 0.09 | 0.58 ± 0.10  | 2.19 ± 0.17 | 0.82 ± 0.10 |
| DGAT2                            | 1.00 ± 0.04 | 0.77 ± 0.07  | 0.99 ± 0.09 | 0.78 ± 0.03 |
| ACC1                             | 1.00 ± 0.05 | 0.89 ± 0.06  | 1.02 ± 0.09 | 0.63 ± 0.06 |
| SCD1                             | 1.00 ± 0.12 | 0.97 ± 0.06  | 1.06 ± 0.10 | 0.29 ± 0.05 |
| PEPCK                            | 1.00 ± 0.11 | 0.86 ± 0.11  | 1.02 ± 0.05 | 1.00 ± 0.08 |
| G6Pase                           | 1.00 ± 0.09 | 0.56 ± 0.07  | 1.15 ± 0.06 | 0.64 ± 0.15 |
| FGF21                            | 1.00 ± 0.20 | 0.81 ± 0.16  | 10.28 ± 1.53 | 3.05 ± 0.94 |
| HMG-CoA synthase                 | 1.00 ± 0.12 | 1.72 ± 0.19  | 1.88 ± 0.22 | 1.42 ± 0.24 |
| Dhcr24                           | 1.00 ± 0.04 | 0.97 ± 0.08  | 1.07 ± 0.06 | 1.04 ± 0.06 |
| L-PK                             | 1.00 ± 0.12 | 0.90 ± 0.04  | 1.77 ± 0.08 | 0.82 ± 0.18 |
| HSL                              | 1.00 ± 0.05 | 0.99 ± 0.07  | 0.94 ± 0.08 | 0.94 ± 0.07 |
| CRBN                             | 1.00 ± 0.05 | 0± 6.21E-04  | 1.91 ± 0.03 | 0± 6.39E-04 |

Data represent the mean ± SEM. *Statistical differences ( *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001)
versus WT mice fed the same diet. *Statistical differences (†*P* < 0.05, ††*P* < 0.01, †††*P* < 0.005, ††††*P* < 0.001)

versus mice with the same genotype that were fed the normal chow diet.