BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-20-215

Title: Gluconeogenic signals regulate hepcidin gene expression via CRBN-KLF15 axis

Article Type: Article

Keywords: Cereblon; Fasting; Gene expression; Hepcidin; Kruppel-like factor 15

Corresponding Author: Yong Deuk Kim

Authors: Jeong-Rang Jo[1,2,#], Sung-Eun Lee[3,#], Seungwon An[4], Balachanda Nedumaran[5], Swati Ghosh[6], Keun-Gyu Park[1,2], Yong Deuk Kim[1,2,3,*]

Institution: [1]Research Institute of Aging and Metabolism, Kyungpook National University,
[2]Department of Internal Medicine, Kyungpook National University Hospital,
[3]Department of Applied Biosciences, Kyungpook National University,
[4]Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago,
[5]Barbara Davis Center for Diabetes and [6]Department of Pediatrics, University of Colorado Anschutz Medical Campus,

Manuscript Type: Article

Title: Gluconeogenic signals regulate hepcidin gene expression via CRBN-KLF15 axis

Author's name: Jeong-Rang Jo[1,2#], Sung-Eun Lee[3#], Seungwon An[4], Balachandar Nedumaran[5], Swati Ghosh[6], Keun-Gyu Park[1,2], Yong Deuk Kim[1,2,3*]

Affiliation: [1]Research Institute of Aging and Metabolism, Kyungpook National University, Daegu 41566, Republic of Korea

[2]Department of Internal Medicine, School of Medicine, Kyungpook National University, Kyungpook National University Hospital, Daegu 41944, Republic of Korea

[3]Department of Applied Biosciences, Kyungpook National University, Daegu 41566, Republic of Korea

[4]Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

[5]Barbara Davis Center for Diabetes, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[6]Department of Pediatrics, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[#]These authors contributed equally to this article as first authors.

Running title: Gluconeogenic signals regulate hepcidin expression

Keywords: Cereblon, Fasting, Gene expression, Hepcidin, Kruppel-like factor 15

***Corresponding Author's Information:**

Yong Deuk Kim, Tel.: 82-53-950-7768, Fax: 82-53-953-7233, E-mail: ydkim94@knu.ac.kr

ABSTRACT

Hepcidin (HAMP) is synthesized in the liver and characterizes the key iron-regulatory hormone that controls systemic iron homeostasis. Cereblon (CRBN) and Kruppel-like factor 15 (KLF15) are known to regulate diverse physiological functions. In this study, we investigate the role of CRBN on hepatic hepcidin gene expression and its production under gluconeogenic stimuli. Fasted mice as well as forskolin (FSK)- and glucagon (GLU)-treated mice had reduced serum iron levels and increased expressions of hepatic *Crbn* and *Klf15* and hepcidin secretion. Fasted and Ad-*Crbn*-infected mice exhibited significant reduction in microRNA-639 (miR-639), but not miR-204 and miR-455. Hepatic overexpression of *Crbn* elevated hepcidin expression and its production along with *Klf15* gene expression, whereas knockdown of *Crbn* and *Klf15* markedly decreased the FSK- and fasting-mediated induction of hepcidin gene expression and its biosynthesis in mouse livers and primary hepatocytes. Moreover, expression of KLF15 significantly increased the hepcidin reporter gene activity, and it was exclusively dependent on the KLF15-binding site identified within the hepcidin gene promoter. Overall, this study demonstrates that CRBN and KLF15 are novel mediators of gluconeogenic signal-induced hepcidin gene expression and its production. CRBN and KLF15 may be novel potential therapeutic targets to intervene the metabolic dysfunction.

INTRODUCTION

Hepcidin, encoded by the HAMP gene, is known to be responsible for iron homeostasis in various mammals (1). It has been demonstrated that targeted disruption of hepcidin in the liver reiterates the hemochromatosis phenotype accompanied by increased plasma iron and massive parenchymal iron accumulation (2). Hepcidin-deficient mouse models appeared to be resistant to tissue damage as well as to dysfunction during pulmonary iron overload with normal expression of upstream stimulatory factor 2 (USF2) (3, 4). Ceruloplasmin, which is involved in iron metabolism, is deregulated in hepcidin-deficient mice (5). Conversely, mouse models of hepcidin transgenic overexpression in the liver demonstrated severe iron-deficiency anemia (6). It has also been shown that hepcidin prevents iron overload and improves erythropoiesis in a mouse model of β-thalassemia (7). Hepatic hepcidin production is increased due to infection, inflammation, and severe iron overload, whereas it is decreased by hypoxia, iron-deficiency anemia, erythropoietin, and growth factors (8). Our recent study showed that the b-cell translocation gene 2 (BTG2)-yin yang 1 (YY1)-hepcidin signaling network controls mammalian iron homeostasis in the liver (8). The present study was aimed to identify novel pathways associated with iron metabolism and gluconeogenesis.

Cereblon (CRBN) is a 442-amino acid protein exhibiting multiple functions in the human brain and other tissues. It was identified as a key gene associated with memory and

learning (9). CRBN is identified as an important molecular target of thalidomide (an antiemetic agent)-mediated teratogenicity (10). CRBN is predominantly expressed in the brain and it is moderately expressed in diverse tissues. It is a component of an E3 ubiquitin ligase complex by interacting with damage-specific DNA-binding protein 1 (DDB1), cullin 4 (Cul4), and regulator of cullins 1 (ROC1), ring box protein 1 (RBX1). Moreover, CRBN directly interacts with large-conductance calcium-activated potassium channels (BK_{Ca}), voltage-gated chloride channel (ClC)-2, ikaros zinc finger (IKZF)1, IKZF3, and AMP-activated protein kinase (AMPK) (9-11). It plays a vital role in the regulation of ion transport and negatively modulates the AMPK signaling pathway *in vivo* and *in vitro* (12) and its metabolism (13). Studies have shown that *Crbn*-deficient mice have hyperphosphorylated AMPK in the livers when fed with normal diet. These mice were significantly protected against weight gain when they were fed with high-fat diet, and they displayed improved insulin sensitivity (13, 14). A recent study reported that CRBN inhibits the activation of AMPK *in vivo* and *in vitro* through direct binding with the AMPK subunit (12). AMPK is an energy sensor at the cellular level by increasing the ATP-producing catabolic pathways and suppressing the ATP-consuming anabolic pathways (15). CRBN regulates AMPK *in vitro*, and *Crbn* deficiency preserves metabolic homeostasis by enhancing AMPK activity in alcoholic liver disease (16).

Kruppel-like factor 15 (KLF15) is a member of the KLF transcription factor family, which contains a zinc finger DNA-binding domain. KLF15 is expressed in diverse tissues such

as the liver, kidney, pancreas, muscle, and heart (17). It is upregulated by glucagon and glucocorticoids during starvation or under diabetic conditions, whereas feeding state and insulin downregulate the expression of KLF15 (18, 19). KLFs are also involved in fibrosis, obesity, cardiovascular disease, cancer, and inflammatory conditions. KLF15 is a key positive regulator of gluconeogenesis and a negative regulator of cardiac hypertrophy and fibrosis (18, 20). However, the critical role of KLF15 in the regulation of iron metabolism through hepcidin gene expression remains unexplored.

In this study, we demonstrated that fasting state and FSK treatment significantly elevated hepatic hepcidin gene expression and its production by increasing the expression of CRBN and KLF15. Moreover, disruption of CRBN and KLF15 markedly decreased hepcidin gene expression and eventually hepcidin secretion under FSK treatment. Our study findings suggest that CRBN and KLF15 are mediators of fasting-induced hepatic hepcidin expression and its biosynthesis. Therefore, targeting CRBN and KLF15 might be a therapeutically important strategy to combat metabolic dysfunction.

RESULTS

Fasting state and forskolin treatment increase hepatic hepcidin metabolism

Gluconeogenic stimuli are known to regulate iron metabolism through hepcidin induction in starving mice (21). Cereblon (CRBN) deficiency is known to prevent diet-induced obesity and insulin resistance by increasing AMPK activation (13). We analyzed the expression of genes in the livers of fasted mice to explore the link between *Crbn*, *Klf15*, and hepcidin under fasting state. We observed that hepatic CRBN, KLF15, and HAMP gene expressions were significantly increased in the fasted mice group compared to those in the non-fasted group (Fig. 1A). Furthermore, we evaluated the expression of microRNAs during fasting state. MicroRNA-639 (miR-639) expression was specifically decreased in the fasted mice, whereas the expression of miR-204 and miR-455 were unchanged in fasted mice (Fig. 1B). Overall, these results suggest that CRBN and miR-639 play a key role in the regulation of hepatic hepcidin gene expression under fasting state. Consistent with *Hamp* gene expression, serum hepcidin levels were significantly elevated in the fasted mice compared to those in mice in the fed state (Fig. 1C), indicating the role of hepcidin during gluconeogenesis. Next, we explored hepcidin expression in response to gluconeogenic stimuli (forskolin and glucagon), and found that forskolin (FSK) and glucagon (GLU) treatment increased the expressions of *Crbn*, *Klf15*, and *Hamp* in mouse livers (Fig. 1F). Similarly, FSK and GLU exposure elevated serum hepcidin levels compared to the control groups (Fig. 1G). However,

serum iron levels were markedly reduced in these four groups (fasted, Hepc25- and FSK- and GLU-treated) compared to their respective control groups (Fig. 1D, E, and H). Taken together, these results suggest an important link between CRBN and hepcidin in the regulation of iron metabolism in response to gluconeogenic stimuli.

CRBN increases hepatic hepcidin gene expression

We investigated the crucial role of *Crbn* as a key positive regulator of hepcidin gene expression. Using an adenoviral delivery system expressing *Crbn* (Ad-*Crbn*) or a control green fluorescent protein (Ad-GFP), we observed that *Crbn* was ectopically expressed in primary mouse hepatocytes. Ad-*Crbn* was successfully overexpressed in primary mouse hepatocytes. Transduction of Ad-*Crbn* significantly enhanced both mRNA and protein levels of KLF15 and HAMP compared to those in Ad-GFP control groups (Fig. 2A and B). Lenalidomide-induced CRBN is known to regulate argonaute 2 (AGO2) protein and its target microRNAs in multiple myeloma cells (22). Hence, we sought to investigate whether *Crbn* has any role in the regulation of microRNAs in mouse livers. MiR-639 expression was specifically decreased by the administration of Ad-*Crbn*, whereas miR-204 and miR-455 expression were not changed in Ad-*Crbn*-infected mice (Fig. 2C). Overall, these results suggest that CRBN can control hepatic hepcidin gene expression via the upregulation of KLF15 and the downregulation of miR-639. Conversely, we examined whether *Crbn*

knockdown could modulate FSK- and fasting-mediated induction of *Klf15* and hepcidin metabolism in primary mouse hepatocytes and in mouse livers using adenoviral knockdown methods. As shown in Fig. 2D-F, the expression of CRBN, KLF15, and HAMP genes were significantly upregulated by FSK treatment and fasting state, and this phenomenon was markedly reduced by *Crbn* knockdown. Interestingly, the increase in serum hepcidin levels induced by fasting state was decreased by silencing of *Crbn*, but not in serum iron levels (Fig. 2G). Collectively, these findings suggest that CRBN mediates the induction of hepcidin gene by FSK treatment.

KLF15 regulates hepcidin gene expression

To understand the effects of KLF15 on hepcidin gene expression and its production, we modulated the hepatic hepcidin gene expression using an adenoviral delivery system in primary mouse hepatocytes. As shown in Fig. 3A, *Klf15* was successfully delivered in primary mouse hepatocytes and also significantly elevated the *Hamp* gene expression. Especially, overexpression of Ad-*Klf15* increased hepcidin amounts compared to those in the control groups (Fig. 3B). We further investigated the more direct effect of KLF15 on FSK- and fasting-induced hepcidin gene expression and its production using adenovirus-mediated silencing of *Klf15* (Ad-sh*Klf15*) *in vivo* and *in vitro*. The expression of *Klf15* in control and FSK-treated primary mouse hepatocytes was effectively repressed by Ad-

sh*Klf15*. The FSK- and fasting-mediation induction of *Hamp* gene expression was dramatically attenuated by *Klf15* knockdown (Fig. 3C and E). Consistent with *Hamp* gene expression, the FSK- and fasting-induced serum hepcidin amounts were prominently decreased by knockdown of endogenous *Klf15*, but not the serum iron levels (Fig. 3D and F). Overall, these observations strongly suggest that KLF15 regulates hepatic hepcidin gene expression and its production.

KLF15 is recruited to the hepcidin promoter

Next, we explored whether KLF15 can directly control the transcriptional activity of hepcidin induced by FSK treatment. We observed that FSK treatment or transiently expressed *Klf15* significantly elevated the *Hamp* promoter activity (Fig. 4A). Our *in silico* analysis, we could predict a KLF15-binding site on the *Hamp* promoter and mutated this binding site on the *Hamp* promoter. A reporter gene assay indicated that FSK treatment and *Klf15* transfection increased the *Hamp* promoter activity, whereas this phenomenon was strikingly abolished in the KLF15-binding site-mutated (mt) *Hamp* gene promoter compared to the wild-type (wt) promoter group (Fig. 4B). To further examine the recruitment of KLF15 protein to the *Hamp* gene promoter and the effect of CRBN on KLF15 recruitment, we performed chromatin immunoprecipitation (ChIP) assay using KLF15 antibody in primary mouse hepatocytes. There was no recruitment of KLF15 protein in the

distal (Dis) region of the promoter where we did not find any KLF15-binding site from the *in silico* analysis. As anticipated, endogenous KLF15 occupancy on the proximal (Pro) region with the KLF15-binding site was significantly increased by FSK treatment compared to that in control groups (Fig. 4C). Overall, these results strongly suggest that KLF15 is recruited on the hepcidin gene promoter region to enhance the transcription of hepcidin gene.

DISCUSSION

Using *in vivo* and *in vitro* experiments, we demonstrated that CRBN enhances hepatic hepcidin gene expression and its secretion by upregulating KLF15 expression. We found that gluconeogenic signals remarkably induced the transcription and production of the hepatic hepcidin gene through the stimulation of the CRBN-KLF15 signaling pathway. In contrast, the stimulatory effects of fasting condition or FSK treatment on hepatic hepcidin gene expression and its secretion were markedly decreased by the knockdown of endogenous *Crbn* and *Klf15* in primary mouse hepatocytes. These findings indicate that the induction of hepatic hepcidin production by the fasting state is KLF15-dependent. Our results suggest that the gluconeogenic stimuli-CRBN-KLF15 signaling network manifests a potent molecular mechanism underlying the regulation of hepcidin gene expression and its production under both *in vivo* and *in vitro* conditions.

Studies have reported that gluconeogenic signals modulate hepatic hepcidin gene expression and its biosynthesis in mice (21, 23, 24). Hepcidin is an established modulator of iron metabolism, inflammation, hypoxia, and erythropoiesis (25, 26). During fasting conditions, the BTG2-KLF15 signaling network modulates the biosynthesis of hepatic fibroblast growth factor 21 (17). Our previous study showed that AMPK was negatively regulated by CRBN in mice with alcoholic liver disease (16). However, there is no

information regarding the potential role of CRBN in modulating hepatic hepcidin gene expression and its secretion. Our current results demonstrated that elevated CRBN expression by the fasting state or FSK treatment controls the production of hepatic hepcidin by promoting *Klf15* expression. The fasting state and FSK challenge enhanced the expression of hepatic *Crbn*, *Klf15*, and *Hamp* gene and increased the amount of serum hepcidin in mice, but not the levels of iron (Fig. 1). Transduction of Ad-*Crbn* efficiently elevated hepatic hepcidin gene expression and its production via the induction of KLF15 expression, whereas this phenomenon was prominently abolished in *Crbn* silenced cells (Fig. 2). These findings suggest that CRBN plays a vital role in regulating the fasting- and FSK-induced hepatic hepcidin gene expression and its production by inducing KLF15 expression.

Glucagon and glucocorticoids are known to induce the transcription factor KLF15 during the fasting state, whereas insulin is known to downregulate KLF15 expression under fed conditions (18-20). It is known that KLF15 modulates fibrosis, obesity, cardiovascular disease, cancer, and inflammatory conditions. Based on this information, we further characterized the novel molecular mechanisms underlying the fasting-mediated induction of hepcidin gene transcription by the CRBN-KLF15 signaling pathway in hepatocytes. As depicted in Fig. 3, KLF15 positively regulated hepcidin gene expression and its production in primary mouse hepatocytes during FSK treatment. For the first time, we identified the

KLF15-binding site on the *Hamp* gene promoter, and the *Hamp* promoter activity was also efficiently increased by the transient transfection of *Klf15* (Fig. 4). Overall, our results suggest that the CRBN-KLF15 signaling pathway mediated the induction of hepcidin gene by gluconeogenic signals.

Xu et al. demonstrated that lenalidomide-induced cell growth and survival are involved in the regulation of CRBN-downstream binding protein AGO2, an essential protein for microRNA maturation. Lenalidomide significantly induced CRBN, subsequently altering the expression of both AGO2 protein and its target microRNAs in multiple myeloma cells (22). Our results demonstrated that miR-639 expression was specifically decreased in the fasted mice. Moreover, miR-639 expression was exclusively decreased by the administration of Ad-*Crbn* (Figs. 1 and 2). Interestingly, Ad-*Crbn*-induced *Klf15*, phosphoenolpyruvate carboxykinase 1 (*Pck1*), and glucose-6 phosphatase (*G6pc*) gene expressions were reduced by miR-639 mimics. However, the miR-639 inhibitor did not alter the induction of *Klf15* and gluconeogenic gene by Ad-*Crbn*. Collectively, these results strongly suggest that CRBN-mediated downregulation of miR-639 regulates hepatic gluconeogenic gene expression by inducing KLF15 expression in the liver and primary hepatocytes (data not shown). However, the molecular mechanism of CRBN-microRNA-KLF15 signaling pathway needs to be investigated. Genetically engineered mouse models of *Crbn* and *Klf15* genes would be useful for exploring their physiological role in the liver in relation to metabolic

dysfunction. High-throughput sequencing analysis in these models would also be more valuable to discover various regulators involving CRBN-downstream binding protein AGO2 and microRNAs linked to CRBN-AGO2-KLF15 and/or the CRBN-microRNA-KLF15 signaling network.

Hepcidin is produced due to iron loading and inflammation at a high rate in the liver and is cleared from the circulation by the kidneys to maintain iron balance in the body (1, 7, 27). Hepcidin deficiency results in iron overload (3-5, 28), whereas overloading of hepcidin leads to the development of iron-restrictive anemias, including autoimmune inflammatory disorders, chronic kidney disease, cancers, infections, and inherited iron-refractory iron-deficiency anemia (25, 27, 28). Psychological stress and overload exercise have been reported to reduce serum iron levels and result in the inhibition of erythropoiesis in rats (29, 30). All these studies report that short-term food deprivation and reduced iron levels could lead to psychological stress and depression symptoms in patients with anorexia or other eating disorders. Our results indicate that fasting condition increased hepcidin production and decreased iron levels. Interestingly, hepcidin treatment strikingly reduced serum iron levels in mice (Fig. 1) indicating an inverse correlation between hepcidin and serum iron levels under fasting condition. In addition, iron-mediated hepcidin regulation is associated with the bone morphogenetic protein (BMP)-SMAD pathway, whereas inflammation-mediated regulation is associated with both the interleukin-6 (IL-6)/Janus kinase (JAK)/signal transducer and

activator of transcription signaling axis and the BMP-SMAD pathway (1, 7, 25, 26, 31). Increased expression of hepcidin regulation under fasting state causes decreased serum iron levels via the upregulation of CRBN, KLF15, and HAMP. Therefore, altering the serum hepcidin rate can upregulate its circulating concentration in the fasting state and consequently change the serum iron flow.

In conclusion, our current study demonstrates that hepcidin is a possible novel target of CRBN, and that CRBN encourages hepatic hepcidin gene expression and its production by inducing KLF15 expression when exposed to gluconeogenic signals such as fasting state or FSK treatment. We speculated that the increase in CRBN expression induced by gluconeogenic stimuli efficiently modulates hepatic hepcidin metabolism by enhancing the expression of KLF15. Furthermore, upregulated CRBN decreases the serum iron levels under fasting state (Fig. 4D). This novel molecular mechanism involving hepatic hepcidin metabolism by the CRBN-KLF15 signaling network may provide better understanding to develop potential therapeutic agents and important strategies to intervene the metabolic dysfunction caused by iron-deficiency anemia.

MATERIALS AND METHODS

Additional detailed methods are described in the Supplementary information.

ACKNOWLEDGMENTS

This research was supported by Kyungpook National University Development Project Research Fund, 2018 (to Y.D.K.).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Fasting and forskolin improves hepatic hepcidin gene expression and its production in mice. (A) Wild-type (WT) mice were fed *ad libitum* (Fed) and fasted for 24 hr (Fast). Gene expressions were analyzed by qPCR using gene-specific primers. Various protein levels in liver tissue extracts were analyzed by immunoblotting using specific antibodies. **(B)** Expression profiles of predicted microRNAs (miR) in mouse livers. **(C)** Serum hepcidin and **(D)** iron contents in the indicated mice groups. **(E)** Serum iron concentration after hepcidin-25 (Hepc25, 100 μg/kg) treatment for 12 h. **(F)** WT mice were injected intraperitoneally with forskolin (FSK, 5 mg/kg) and glucagon (GLU, 10 μg/kg) for 6 hr. Then, qPCR experiments were performed using various primers. **(G)** Serum levels of hepcidin and **(H)** iron at the indicated condition. n = 5 mice per group. $*P < 0.05$, $**P < 0.01$ vs. fed mice or untreated control mice.

Figure 2. CRBN elevates hepatic hepcidin gene expression in mouse primary hepatocytes. (A) Mouse primary hepatocytes were infected with Ad-GFP and Ad-*Crbn* at a multiplicity of infection of 60 for 36 hr. Gene expression was analyzed by qPCR using various primers. **(B)** Analysis of various protein levels in mouse primary hepatocytes was conducted using the corresponding antibodies used for immunoblotting. **(C)** The expression of miR-204, miR-455, and miR-639 were quantified by qPCR analysis. **(D)** Mouse primary hepatocytes were infected

with Ad-Scramble (Ad-Scram) and Ad-sh*Crbn* at a multiplicity of infection of 60 for 36 hr. After infection, the cells were treated with FSK (10 μM) for 12 hr. Total RNAs were utilized for qPCR analysis using gene-specific primers. **(E)** Whole-cell extracts were harvested from the indicated groups of mouse primary hepatocytes and immunoblotted with specific antibodies. **(F)** WT mice were infected with Ad-Scram and Ad-sh*Crbn* for 7 days. Then, these mice were fasted for 24 hr. Total RNAs were utilized for qPCR analysis using gene-specific primers. **(G)** Serum levels of hepcidin and iron at the indicated condition. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. untreated control cells, Ad-GFP, FSK-treated cells, fed or fasted mice.

Figure 3. KLF15 regulates hepatic hepcidin gene expression and its production in mouse primary hepatocytes. (A) Mouse primary hepatocytes were infected with Ad-GFP and Ad-*Klf15* at a multiplicity of infection of 60 for 36 hr. Gene expressions were analyzed by qPCR using the indicated primers. **(B)** Hepcidin concentration in the observed mouse primary hepatocytes. **(C)** Mouse primary hepatocytes were infected with Ad-Scram and Ad-sh*Klf15* at a multiplicity of infection of 60 for 36 hr, and then treated with FSK for 12 hr. Total RNAs were utilized for qPCR analysis using gene-specific primers. **(D)** Hepcidin concentration under indicated conditions. **(E)** WT mice were infected with Ad-Scram and Ad-sh*Klf15* for 7 days. The mice were then fasted for 24 hr. Quantitative PCR analysis shows *Hamp* expression in the liver. **(F)** Levels of serum hepcidin and iron under the observed condition. $*P < 0.05$, $**P <$

0.01, and ***$P < 0.001$ vs. untreated controls, Ad-GFP, FSK-treated cells, fed or fasted mice.

Figure 4. KLF15 is an important regulator of hepcidin gene transcription in hepatocytes. **(A)** AML-12 cells were transiently transfected with *Klf15* and the indicated reporter gene for 36 hr, and then treated with FSK for 12 hr. **(B)** AML-12 cells were cotransfected with wild-type (wt) and mutant (mt) forms of the *Hamp* gene promoter and *Klf15* for 36 hr. After transfection, the cells were treated with FSK for 12 hr. **(C)** Chromatin immunoprecipitation (ChIP) assay for the recruitment of KLF15 on the *Hamp* gene promoter. Mouse primary hepatocytes were infected with Ad-Scram and Ad-sh*Klf15* for 36 hr, and then treated with FSK for 12 hr. Cell lysates were immunoprecipitated with anti-KLF15 antibody. Purified DNA samples were employed for PCR using specific primers binding to the specific proximal (Pro) and nonspecific distal (Dis) regions on the *Hamp* gene promoter. Input was 10% of the soluble chromatin. (D) Schematic model illustrating the regulation of hepcidin gene expression and its production. Fasting state and forskolin treatment enhance hepatic hepcidin gene expression by stimulating the CRBN-KLF15 signaling network, which subsequently controls iron metabolism via the increase in hepcidin output and the decrease in iron production. *$P < 0.05$ vs. untreated control.

REFERENCES

1. Anderson ER and Shah YM (2013) Iron homeostasis in the liver. Compr Physiol 3, 315-330

2. Zumerle S, Mathieu JR, Delga S et al (2014) Targeted disruption of hepcidin in the liver recapitulates the hemochromatotic phenotype. Blood 123, 3646-3650

3. Lesbordes-Brion JC, Viatte L, Bennoun M et al (2006) Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis. Blood 108, 1402-1405

4. Deschemin JC, Mathieu JRR, Zumerle S, Peyssonnaux C and Vaulont S (2017) Pulmonary Iron Homeostasis in Hepcidin Knockout Mice. Front Physiol 8, 804

5. Viatte L, Lesbordes-Brion JC, Lou DQ et al (2005) Deregulation of proteins involved in iron metabolism in hepcidin-deficient mice. Blood 105, 4861-4864

6. Nicolas G, Bennoun M, Porteu A et al (2002) Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci U S A 99, 4596-4601

7. Ganz T and Nemeth E (2012) Hepcidin and iron homeostasis. Biochim Biophys Acta 1823, 1434-1443

8. Lee SE, Hwang SL, Jang WG, Chang HW and Kim YD (2015) B-cell translocation gene 2 promotes hepatic hepcidin production via induction of Yin Yang 1. Biochem Biophys Res Commun 460, 996-1001

9. Shi Q and Chen L (2017) Cereblon: A Protein Crucial to the Multiple Functions of Immunomodulatory Drugs as well as Cell Metabolism and Disease Generation. J Immunol

Res 2017, 9130608

10. Chang XB and Stewart AK (2011) What is the functional role of the thalidomide binding protein cereblon? Int J Biochem Mol Biol 2, 287-294

11. Kim HK, Ko TH, Nyamaa B et al (2016) Cereblon in health and disease. Pflugers Arch 468, 1299-1309

12. Lee KM, Yang SJ, Choi JH and Park CS (2014) Functional effects of a pathogenic mutation in Cereblon (CRBN) on the regulation of protein synthesis via the AMPK-mTOR cascade. J Biol Chem 289, 23343-23352

13. Lee KM, Yang SJ, Kim YD et al (2013) Disruption of the cereblon gene enhances hepatic AMPK activity and prevents high-fat diet-induced obesity and insulin resistance in mice. Diabetes 62, 855-1864

14. Lee KM, Jo S, Kim H, Lee J and Park CS (2011) Functional modulation of AMP-activated protein kinase by cereblon. Biochim Biophys Acta 1813, 448-455

15. Foretz M and Viollet B (2011) Regulation of hepatic metabolism by AMPK. J Hepatol 54, 827-829

16. Kim YD, Lee KM, Hwang SL et al (2015) Inhibition of cereblon by fenofibrate ameliorates alcoholic liver disease by enhancing AMPK. Biochim Biophys Acta 1852, 2662-2670

17. Kim YD, Hwang SL, Jeon HJ et al (2019) B-cell translocation gene 2 enhances fibroblast growth factor 21 production by inducing Kruppel-like factor 15. Sci Rep 9, 3730

18. McConnell BB and Yang VW (2010) Mammalian Kruppel-like factors in health and diseases. Physiol Rev 90, 1337-1381

19. Teshigawara K, Ogawa W, Mori T et al (2005) Role of Kruppel-like factor 15 in PEPCK gene expression in the liver. Biochem Biophys Res Commun 327, 920-926

20. Pearson R, Fleetwood J, Eaton S, Crossley M and Bao S (2008) Kruppel-like transcription factors: a functional family. Int J Biochem Cell Biol 40, 1996-2001

21. Vecchi C, Montosi G, Garuti C et al (2014) Gluconeogenic signals regulate iron homeostasis via hepcidin in mice. Gastroenterology 146, 1060-1069

22. Xu Q, Hou YX, Langlais P et al (2016) Expression of the cereblon binding protein argonaute 2 plays an important role for multiple myeloma cell growth and survival. BMC Cancer 16, 297

23. Zhang Y, Wang X, Wu Q et al (2018) Adenine alleviates iron overload by cAMP/PKA mediated hepatic hepcidin in mice. J Cell Physiol 233, 7268-7278

24. Aigner E, Felder TK, Oberkofler H et al (2013) Glucose acts as a regulator of serum iron by increasing serum hepcidin concentrations. J Nutr Biochem 24, 112-117

25. Ruchala P and Nemeth E (2014) The pathophysiology and pharmacology of hepcidin. Trends Pharmacol Sci 35, 155-161

26. Rishi G, Wallace DF and Subramaniam VN (2015) Hepcidin: regulation of the master iron regulator. Biosci Rep 35, e00192

27. Fung E and Nemeth E (2013) Manipulation of the hepcidin pathway for therapeutic purposes. Haematologica 98, 1667-1676

28. Ganz T and Nemeth E (2011) Hepcidin and disorders of iron metabolism. Annu Rev Med 62, 347-360

29. Wei C, Zhou J, Huang X and Li M (2008) Effects of psychological stress on serum iron and erythropoiesis. Int J Hematol 88, 52-56

30. Navas FJ and Córdova A (2000) Iron distribution in different tissues in rats following exercise. Biol Trace Elem Res 73, 259-268

31. Sangkhae V and Nemeth (2017) Regulation of the Iron Homeostatic Hormone Hepcidin. Adv Nutr 8, 126-136

Fig. 1

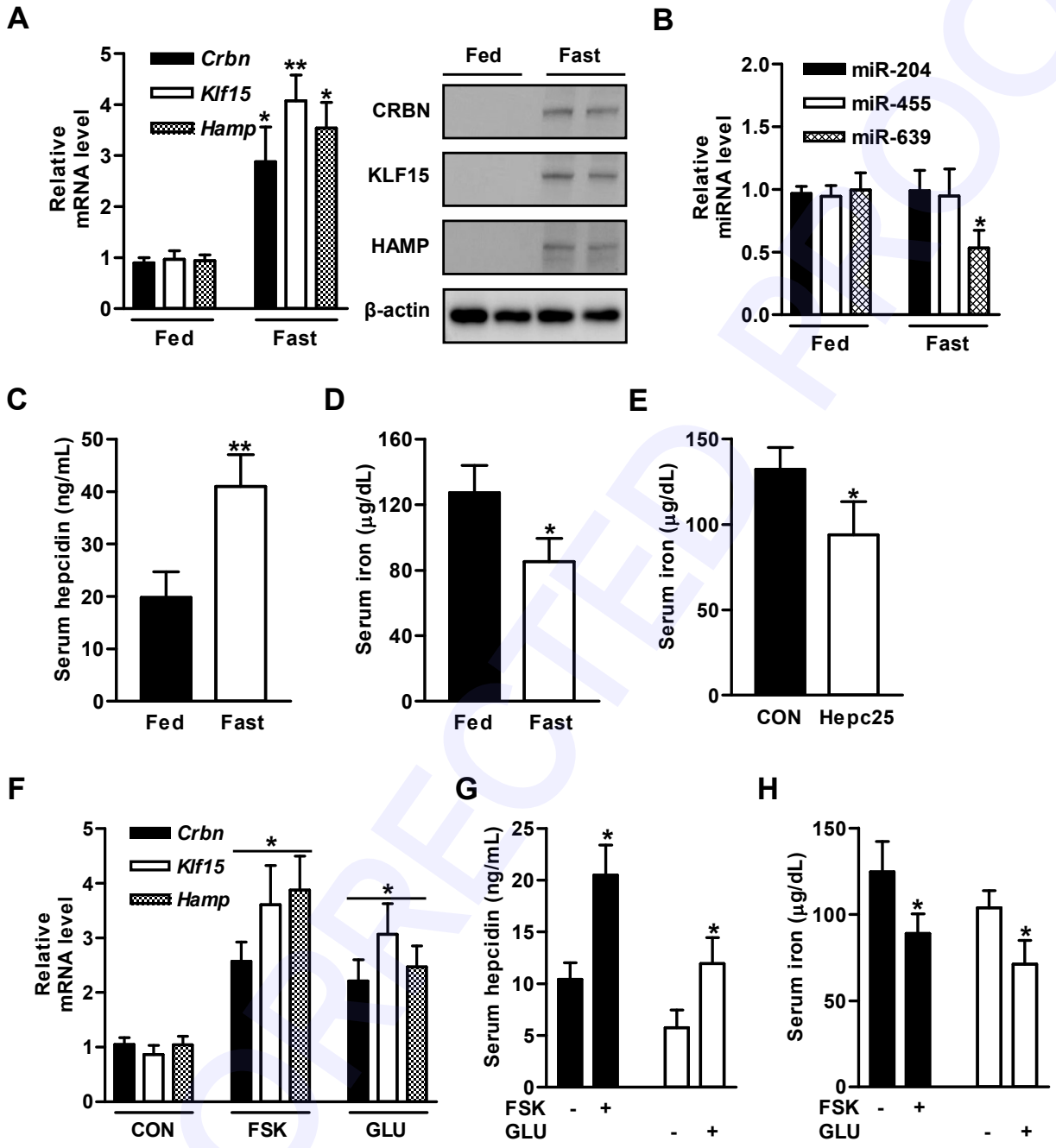


Fig. 2

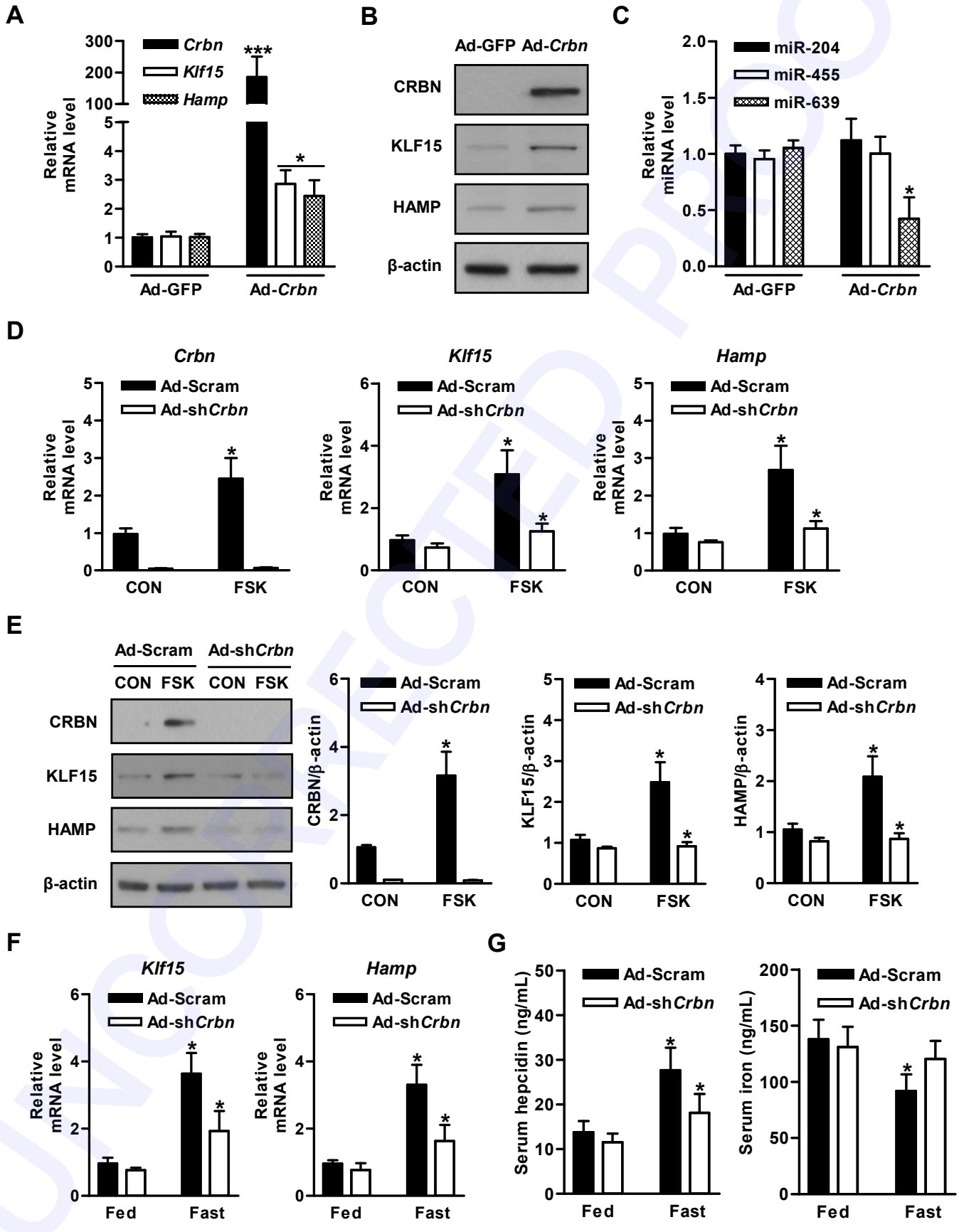


Fig. 3

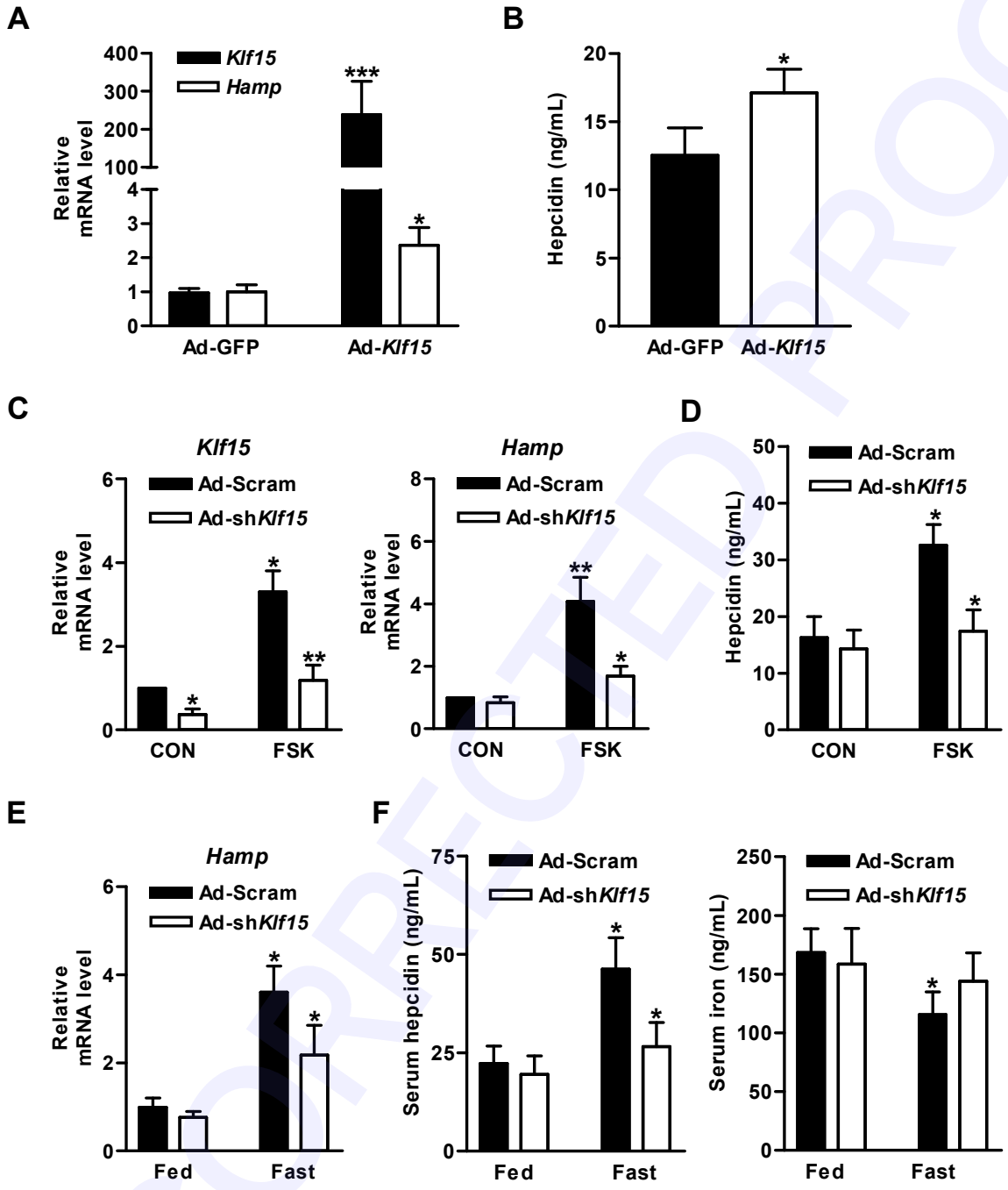
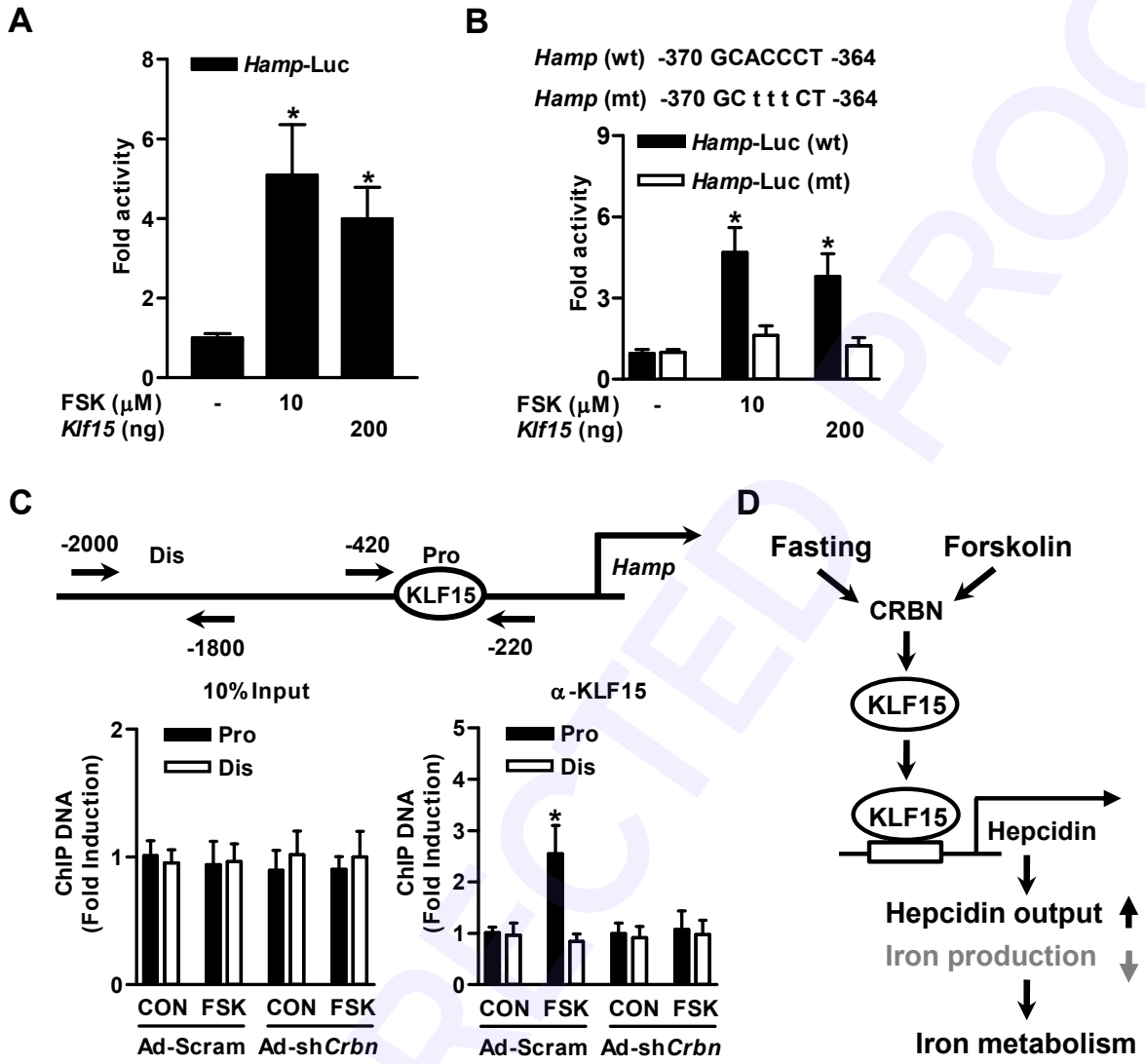


Fig. 4



Title: Gluconeogenic signals regulate hepcidin gene expression via CRBN-KLF15 axis

Authors: Jeong-Rang Jo[1,2#], Sung-Eun Lee[3#], Seungwon An[4], Balachandar Nedumaran[5], Swati Ghosh[6], Keun-Gyu Park[1,2], Yong Deuk Kim[1,2,3*]

Affiliation:

[1]Research Institute of Aging and Metabolism, Kyungpook National University, Daegu 41566, Republic of Korea

[2]Department of Internal Medicine, School of Medicine, Kyungpook National University, Kyungpook National University Hospital, Daegu 41944, Republic of Korea

[3]Department of Applied Biosciences, Kyungpook National University, Daegu 41566, Republic of Korea

[4]Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

[5]Barbara Davis Center for Diabetes, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[6]Department of Pediatrics, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[#]**These authors contributed equally to this article as first authors.**

Running title: Gluconeogenic signals regulate hepcidin expression

Keywords: Cereblon, Fasting, Gene expression, Hepcidin, Kruppel-like factor 15

***Corresponding Author's Information:**

Yong Deuk Kim,

Research Institute of Aging and Metabolism, Department of Internal Medicine, School of Medicine, Department of Applied Biosciences, Kyungpook National University, Daegu 41566, Republic of Korea.

Tel.: 82-53-950-7768, Fax: 82-53-953-7233,

E-mail: ydkim94@knu.ac.kr

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Animal experiments

Male C57BL/6 mice (aged 8 weeks, Samtako, Osan, Republic of Korea) were housed and used in this study. For studies in the fasting and feeding states, mice were fed *ad libitum* (fed) in pathogen-free conditions and then fasted for 24 hr (fast) with free access to water. To examine the potent effect of forskolin (FSK, Sigma-Aldrich, St. Louis, MO, USA) and glucagon (GLU, Sigma-Aldrich), 8-week-old mice were injected intraperitoneally with FSK (5 mg/kg of body weight) or GLU (10 μg/kg of body weight) for 6 hr. For hepcidin (Fisher Scientific, Pittsburgh, PA, USA) administration, wild-type (WT) mice were injected intraperitoneally with recombinant hepcidin-25 (Hepc25, 100 μg/kg) for 12 hr. For the ablation of *Crbn* and *Klf15* experiments, WT mice were intravenously injected with Ad-sh*Crbn* (1×10^9 plaque-forming units, pfu) and Ad-sh*Klf15* (1×10^9 pfu) for 7 days. All animals were sacrificed, and the liver tissues and blood samples were collected. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Kyungpook National University and were conducted according to the rules and guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Measurement of hepcidin and iron levels

Hepcidin levels were measured using a mouse hepcidin ELISA kit (CUSABIO, Wuhan, Hubei Province, China), and iron levels were measured using an iron assay kit (Abcam, Cambridge, MA, USA), according to the manufacturer's instructions (1).

DNA construction

The reporter plasmid containing the mouse *Hamp* promoter and the *Klf15* expression

vector were generously donated by Dr. Hueng-Sik Choi (Chonnam National University, Gwangju, Republic of Korea) and Dr. Myung-Shik Lee (Yonsei University College of Medicine, Seoul, Republic of Korea), respectively (2, 3). The *Klf15* mutant on the mouse *Hamp* luciferase reporter construct was generated using the site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The specific primers we used were as follows: forward, 5′-GGTGATGCTTTCTGCACATG-3′, and reverse, 5′-CATGTGCAGAAAGCATCACC-3′. The plasmid encoding the HA-tagged mouse *Crbn* was kindly provided by Dr. Chul-Seung Park (Gwangju Institute Science and Technology, Gwangju, Republic of Korea). All plasmids were verified by DNA sequencing.

Cell culture and transient transfection assays

AML-12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% insulin-transferrin-selenium mixture (ITS, Gibco-BRL), dexamethasone (40 ng/ml, Sigma-Aldrich), and 1% antibiotics (Gibco-BRL). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Transient transfection assays were conducted using AML-12 cells, as previously described (3, 4). Briefly, transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The total amount of DNA used for each transfection was adjusted to 0.8 μg/well by adding an appropriate amount of empty vector and expression vectors encoding various genes. Cytomegalovirus (CMV)-β-galactosidase plasmids (100 ng) were used as internal control to correct for transient transfection efficiency. The activity of β-galactosidase was used for normalization.

Isolation and culture of primary mouse hepatocytes

Primary mouse hepatocytes were isolated from the livers of 8-week-old male C57BL/6 mice (Samtako) using a portal vein collagenase (Sigma-Aldrich) perfusion method and subsequently cultured in M199 medium (Cellgro, Herndon, VA, USA), as described previously (2, 4).

Recombinant adenovirus

Details regarding the corresponding adenoviruses encoding full-length *Klf15* (Ad-*Klf15*), green fluorescent protein (GFP), and adenoviral delivery system for *Klf15*-targeted shRNA (Ad-sh*Klf15*) have been described previously (3). Ad-*Crbn* and Ad-sh*Crbn* were purchased from Vector Biolabs (Malvern, PA, USA). Primary mouse hepatocytes were infected with adenoviral vectors expressing *Crbn* (Ad-*Crbn*) and *Klf15* (Ad-*Klf15*) at a multiplicity of infection of 60 for 36 hr to overexpress *Crbn* and *Klf15*. Primary mouse hepatocytes were also infected with adenoviral delivery system for *Crbn*- and *Klf15*-targeted short hairpin RNA (shRNA) (Ad-sh*Crbn*) and Ad-sh*Klf15* at a multiplicity of infection of 60 for 36 hr to knockdown *Crbn* and *Klf15*.

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNAs were isolated and purified from liver tissues and primary mouse hepatocytes using the TRIzol method (Invitrogen), as previously reported (2-4). cDNA was generated using the Maxima® First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). It was analyzed by qPCR using an SYBR Green PCR kit (Applied Biosystems, Waltham, MA, USA) and a StepOne™ Real-time PCR system (Applied Biosystems). The levels of *Crbn*, *Klf15*, and *Hamp* mRNA were analyzed by qPCR, as described previously (2, 3, 5). The expression levels of the indicated genes were normalized to ribosomal L32 expression. For microRNAs expression analysis, total RNA was isolated using the miRNeasy

isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The abundance of microRNAs was measured using qPCR analysis, as described previously (6-8). The small nuclear RNA U6 was used to normalize target miRNA expression.

Immunoblotting

Proteins were prepared from primary mouse hepatocytes using a RIPA buffer (Elpis-Biotech, Daejeon, Republic of Korea), and immunoblot analysis was performed as described previously (3, 5, 9). The membranes were probed using CRBN (Cell signaling Technology, Danvers, MA, USA), KLF15, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and HAMP (Abcam) antibodies. After incubation with the indicated antibodies, the blots were visualized using an Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA).

Chromatin immunoprecipitation assay (ChIP)

The ChIP assay was conducted according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA), as previously reported (2, 3). Briefly, primary mouse hepatocytes were crosslinked with 1% formaldehyde and then harvested. Sonicated soluble chromatin was immunoprecipitated with KLF15 antibody (Santa Cruz Biotechnology). The final DNA extractions were quantified by qPCR analysis using specific primers encompassing the proximal region of hepcidin promoter (forward 5′-CTCCAGGGCTAATGCTGACA-3′, reverse 5′-CTCAGAAAAATTCCCCTTCT-3′) and the nonspecific distal region of hepcidin promoter (forward 5′-TGGAAAATGGACAAAAAAT-A-3′, reverse 5′-ATCAGCACCCCAGGGCCTCA-3′).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, CA, USA). Statistical significance of the differences between two groups was estimated using Student's *t*-test, and multiple comparisons were evaluated using one-way ANOVA under treatment and experiment. Results are expressed as mean ± standard error of mean. All *P* values < 0.05 were considered to be statistically significant.

REFERENCES

1. Lee YS, Kim YH, Jung YS et al (2017) Hepatocyte toll-like receptor 4 mediates lipopolysaccharide-induced hepcidin expression. Exp Mol Med 49, e408
2. Lee SE, Hwang SL, Jang WG, Chang HW and Kim YD (2015) B-cell translocation gene 2 promotes hepatic hepcidin production via induction of Yin Yang 1. Biochem Biophys Res Commun 460, 996-1001
3. Kim YD, Hwang SL, Jeon HJ et al (2019) B-cell translocation gene 2 enhances fibroblast growth factor 21 production by inducing Kruppel-like factor 15. Sci Rep 9, 3730
4. Kim YD, Hwang SL, Lee EJ et al (2017) Melatonin ameliorates alcohol-induced bile acid synthesis by enhancing miR-497 expression. J Pineal Res 62, e12386
5. Yang SJ, Jeon SJ, Van Nguyen T, Deshaies RJ, Park CS and Lee KM (2020) Ubiquitin-dependent proteasomal degradation of AMPK gamma subunit by Cereblon inhibits AMPK activity. Biochim Biophys Acta Mol Cell Res 1867, 118729
6. Huo Z, Xu X, Zhou L et al (2017) The long non-coding RNA MALAT1 promotes the migration and invasion of hepatocellular carcinoma by sponging miR-204 and releasing SIRT1. Tumour Biol 39, 1010428317718135
7. Fang S, Feng J, Zhang H et al (2020) MiR-455 targeting SOCS3 improve liver lipid disorders in diabetic mice. Adipocyte 9, 179-188
8. Xiao J, Liu Y, Wu F et al (2020) miR-639 Expression Is Silenced by DNMT3A-Mediated

Hypermethylation and Functions as a Tumor Suppressor in Liver Cancer Cells. Mol Ther 28, 587-598

9. Wang H, Li H, Jiang X, Shi W, Shen Z and Li M (2014) Hepcidin is directly regulated by insulin and plays an important role in iron overload in streptozotocin-induced diabetic rats. Diabetes 63, 1506-1518