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Hrd1-ERAD controls production of the hepatokine FGF21 through CREBH polyubiquitination

Juncheng Wei, Lu Chen, Fei Li, Yanzhi Yuan, Yajun Wang, Wanjun Xia, Yuehui Zhang, Yuanming Xu, Zhao Yang, Beixue Gao, Chaozhi Jin, Johanna Melo-Cardenas, Richard M. Green, Hui Pan, Jian Wang, Fuchu He, Kezhong Zhang and Deyu Fang.

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19th Febuary 2018

Thank you for submitting your manuscript on the post prandial regulation of FGF21 level through HRD1-mediated CREBH degradation. The manuscript has now been reviewed by three expert referees whose comments are provided below.

As you can see, while all referees consider the findings novel and potentially interesting, they also raise some critical points that need to be addressed before they can support publication here.
In particular, referees #1 and #3 are concerned that: i) the physiological relevance of the study is unclear; ii) the unique role of HRD1 in regulating CREBH ubiquitination and FGF21 level is not sufficiently proven; and iii) the finding that HRD1 cytoplasmic tail can degrade CREBH needs to be tested. Another major criticism raised by all the referees relates to K27-mediated ubiquitination of CREBH, for which more insight is requested.

Addressing these issues through decisive additional data as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would thus like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS

Referee #1:

Wei and colleagues explored the function of Hrd1-ERAD in regulating FGF21 through CREBH in the liver. Using a liver-specific Hrd1 knockout mouse model (LKO), they found a growth retardation phenotype and elevated mRNA and circulating levels of FGF21. They further

demonstrated that the lack of degradation of CREBH lead to the increased FGF21 expression and characterized some molecular aspects of the Hrd1-mediated ubiquitination of CREBH.

The study presents a potentially important and interesting mechanism for how FGF21 is regulated post-prandially and it provides a nice physiologic context to explain the role of HRD1 in liver. Much of the data, and in particular the biochemical analysis of HRD1-meditated ubiquitination of CREBH, are very good. However, there are several concerns that would first need to be addressed to support the conclusions. The notion that CREBH links ERAD to FGF21 was not sufficiently proven and the physiological relevance of the study is unclear, as elaborated below. With the appropriate revisions to address the key questions about the physiologic relevance and conclusive link between CREBH and FGF21 biology, I would be highly supportive of this paper for publication.

Major comments:

1. The causal relationship between the CREBH accumulation and the LKO phenotypes should be demonstrated. This is preferably done using AAV-mediated knockdown system (over adenovirus as it triggers inflammation and other side-effects), followed by characterization of some of the FGF21-dependent phenotypes (e.g., growth, activity or female fertility). In addition, a successful knockdown of CREBH protein should be shown by Western blot and not just mRNA.

2. The physiological relevance of the finding was not proven. The study showed an inverse correlation between CREBH and HRD1 during fasting-refeeding and good biochemical data to suggest a connection. However, it is unclear whether the increased HRD1 is the reason for reduced CREBH and FGF21 during refeeding. If this cannot be addressed experimentally, it should be commented on as a caveat to interpreting the results, and the title and abstract changed accordingly. In addition, PPARalpha is known as a major regulator of hepatic FGF21 transcription during fasting-refeeding, with CREBH as a potential cofactor. The relationship between CREBH and PPARa on the Fgf21 promoter in the absence or presence of ERAD as well as PPARa protein levels might be demonstrated to support the conclusions.

3. The authors show that LKO mice exhibit some of the same phenotypes as FGF21 transgenic mice with respect to growth, activity and reproduction. Nevertheless, it would be more compelling to show these animals display some of the key beneficial effects of FGF21 overexpression, such as changes in blood glucose, triglycerides, ketone body levels, and resistance to diet induced obesity, etc. The authors should demonstrate at least of some of these other phenotypes.

4. The authors only showed that Hrd1 is sufficient to degrade CREBH, but not whether it is required for CREBH turnover. This is necessary to explain the elevated CREBH protein level in LKO liver. To this end, CREBH turnover should be examined in Hrd1 KO primary hepatocytes or cells.

5. It is confusing that the cytosolic domain of HRD1 is sufficient to degrade CREBH. The authors showed that only the ER-localized CREBH could interact with HRD1. As HRD1-C lacks the ER-transmembrane domain, how does it degrade CREBH on the ER? The intracellular localization of HRD1-C vs. full length should be compared, and whether HRD1-C degrades cleaved CREBH should be measured. In line with this, the localization of CREBH in the ER and/or nucleus during fasting-feeding and in the LKO liver should be demonstrated.

6. Using Hrd1/Fgf21 double knockout mice, the authors showed that FGF21 is responsible for the phenotypes of LKO mice. The authors should also provide some corollary data at the level of gene expression in the liver. The authors mentioned in the discussion session that the FGF21 deletion only partially rescued body weight loss in the LKO mice. These data should be shown to allow assessment on the data.

Minor comments:

1. The authors observed the activation of PERK-ATF4 branch of UPR in the LKO liver. What happens to the other two branches of UPR? In fact, IRE1a-Xbp1 branch has also been shown to regulate FGF21 in the liver (PMID 25170079). The authors might explore this angle more in depth, although this is not necessary for this first report. In considering this, the effect of PERK inhibitor

should be examined more carefully. ATF4 protein level and the expression of additional ATF4 target genes should be measured to demonstrate the inhibition of PERK-ATF4 activity.
2. Sample size and number of repeats are not described throughout the manuscript. Statistical analyses are needed in Fig 4C, 4I, 4L and 5E.
3. K27 ubiquitination is interesting and unexpected. Is it known whether this a CREBH- or Hrd1-specific phenomenon?
4. In Fig 4H, unlike Fig 4D, the HRD1 and CREBH protein levels seems unaltered during fasting-refeeding. Please explain.
5. The endogenous co-IP in Fig 4F should include LKO liver as a negative control to avoid non-specific binding of CREBH to HRD1 antibody.
6. Some figure legends and panels were not cited correctly in the text. Please double check. There are two Fig 3 legends and no Fig 4 legend, and Fig 3H, S3E, 4G (CA mutant) were not mentioned in the text.
7. A thorough editing of English and grammar usage are needed.

Referee #2:

In their manuscript "Hrd1-ERAD controls the hepatokine FGF21 production through K27-linked polyubiquitination of CREBH" Jucheng Wei et.al. address the metabolic function of the E3-ligase HRD1. Through an RNA sequencing experiment the authors identified FGF21 to be upregulated in a liver-specific HRD1 knockout mouse (HRD1 LKO). The phenotypes of the HRD1 LKO mice phenocopy the FGF21 overexpression mouse line, such as female infertility, circadian behavior disruption and bone loss.
Through a proteomics approach the authors further identified CREBH, which is a known transcription factor of FGF21, to be a target of HRD1. This finding was further verified by the authors through Co-Immunoprecipitation experiments and ubiquitylation assays. Additionally, they were able to show that only the full-length protein can interact with HRD1. Furthermore, they identified that CREBH is marked for degradation by an unusual K27-linked poly-ubiquitylation of the lysine 294 by HRD1. This missing regulation of CREBH in the liver-specific Hrd1 knockout mice leads to the upregulation of FGF21 which further leads to female infertility, circadian behavior disruption and bone loss. The phenotypes of the HRD1 LKO mice could be rescued by a knockdown of FGF21 by shRNA.

The data shown in this manuscript presents a metabolic function of the HRD1 E3-ligase.The data suggests a transcriptional regulation of FGF21 in the liver to control a crosstalk with multiple distal organs to control female fertility, circadian rhythm and growth. This work describes a so far uncharacterized role of HRD1, showing a new role of HRD1 in metabolic processes, which will definitely attract attention of the broad readership of EMBO J.

Major Comments:
• Fig 1A and S1A-B: in the text it is referred to that the authors identified Hrd1 in the proteomics experiment, however, Hrd1 is not listed in any of the named figures.
• In the text the authors claim that they define a novel type of poly-ubiquitin chain for ERAD, however they never looked at other factors involved in ERAD. It could be that Hrd1 acts independent of the canonical ERAD pathway. This point is very important and needs further clarification.
• Fig 4G: The Poly-ubiquitin Blot is not very convincing. The degreeof polyubiquitination looks artificial? Maybe an additional full Myc-blot or Coomassie Gel would shed some light onto this poly-ubiquitination pattern.
Minor Comments:
• Fig 1A: The labeling is not clear, what is shown in lane 1 and what in lane 2?
• Fig 4: Wrong label, is labeled as Fig 3.
• Fig 6B: There is a minus missing in the Blot labeling.
• Fig 7: The model figure needs some more graphical clarity.

Referee #3:

In this manuscript, the authors describe the phenotypes of mice with conditional deletion of Hrd1 in the liver. These mice are smaller and the females are infertile. The phenotypes are similar to those of Fgf21 overexpressing mice. The authors then show that Fgf21 levels are increased and suggest that Hrd1 targets the degradation of Crebh, a ER-localized transcription factor for Fgf21. Finally, Fgf21 deletion rescues some of the phenotypes observed in mice with liver deletion of Hrd1.

Overall the study is interesting. It seems reasonable to postulate that Hrd1 targets Crebh since Hrd1 has been reported to target similar transcription factors in the same family including Oasis and Bbf2h7. However, direct demonstration of Crebh degradation by Hrd1 needs to be demonstrated. The authors rely on transfection of Hrd1 to show increased degradation of transfected Crebh. It will be more convincing to show that Crebh degradation is impaired in cells lacking Hrd1. This can be verified in hepatocytes or MEFs, for example. The requirement for Hrd1 in Crebh degradation should at least be demonstrated in cells with Hrd1 knockdown or knockout with appropriate reconstitution controls.

Crebh is one of many transcription factors regulating the expression of Fgf21. Although the authors are to be applauded for showing that deletion of Fgf21 rescues some of the phenotypes, it is not clear that increased Crebh is the sole reason for the increased Fgf21 in Hrd1 liver knockouts.

The authors try to emphasize the importance of K27 polyubiquitin chains in the regulation of Crebh. Since Crebh is presumbly degraded by Hrd1 ubiquitination, it is not clear what the significance of K27 linkage is in this model. However, if the authors want to establish a role for K27 ubiqutination, more data will have to be provided. Based on the model suggested by the authors, Crebh is ubiquitinated predominantly, if not exclusively, with K27 chains. In this case, single lysine mutants of ubiquitin will be dominant negative except for the mutant with K27 only. The data in Fig 5 seems to support this idea. However, there are variations, which probably reflect different expression levels of these mutants. This should be shown in the figure to allow assessment of the effects of these mutants on Crebh ubiquitination. More importantly, the authors show investigate the effects of expressing K27R mutant. If the proposed model is correct, this mutant should inhibit ubiquitination and degradation of Crebh in Hrd1 expressing or overexpressing cells.
Similar experiments should be performed looking at ubiquitination of Crebh in single K to R mutants of Ub. Hrd1 can work with a number of E2s, none of which has been shown to make exclusively K27 chains. Is there another E3 involved?

The authors report that the cytoplasmic tail of Hrd1, without the transmembrane region, is enough to degrade Crebh. However, the transmembrane region of Hrd1 is required for Hrd1 interaction with Crebh. The authors should provide some insights into these observations. Will overexpressing another E3 achieve increase the degradation of Crebh?

Other comments:

The manuscript is poorly written. There is not enough information provided about the Methods used in this study. For example, the authors refer to an earlier paper describing the Hrd1 knockout mice but fail to provide a reference. It is important to describe the exons and domains of Hrd1 deleted in this model. In addition, it is important to confirm that no dominant negative truncation mutant is expressed. It is also not clear what cells are used in the different figure panels. The figure legends should provide enough information for readers to understand the experiments.

The authors provide some description of their proteomics method. However, it is not clear if these are standard procedures or novel implementations. The authors claim that they have developed a method for measuring protein levels by spectral counting. However, without rigorous comparison to isotope labeling, it is not possible to know if their method is reliable. Simply showing the measurements are consistent between samples is not sufficient to guarantee the measurements are accurate. Variance can be reduced by data transformation, for example, such as log transform used in this study. In addition, the authors pad missing values with the lowest values of each replicate. What is the effect of this procedure on the statistics derived from the data?

Most of Figures 1, 2 and 3 belong to supplemental data.

Molecular weight markers should be shown throughout.

We appreciate the efforts for you and all the three reviewers in reviewing our manuscript and their recognition of the significance of our study. All the concerns have been carefully addressed in a point-to-point manner as indicated in this rebuttal letter as well as in the revised manuscript. New figures added to the revised manuscript include Fig. 2H-I, Fig. EV2E-H, Fig. EV3, Fig. 4E&J, Fig. EV4 F&H, Fig. EV5 A-C, Fig. 6, Fig. EV6, Fig. 7B&E and Fig. EV7 . Changes made to the manuscript are underlined.

Referee #1:

Major comments:

1. The causal relationship between the CREBH accumulation and the LKO phenotypes should be demonstrated. This is preferably done using AAV-mediated knockdown system (over adenovirus as it triggers inflammation and other side-effects), followed by characterization of some of the FGF21-dependent phenotypes (e.g., growth, activity or female fertility). In addition, a successful knockdown of CREBH protein should be shown by Western blot and not just mRNA.

Reply: We agree with the reviewer that adenovirus mediates short-term expression and it potentially triggers inflammation and other side effects. To further validate our conclusion, we generated Adeno-associated viral *shCrebh* and administrated to HRD1 LKO mice, which dramatically decreased CREBH expression in the liver of HRD1 LKO mice **(Fig. 6A & B)**. Consistent with our initial observation, CREBH knockdown resulted in a significant decrease in both mRNA and protein levels of FGF21 in the HRD1 LKO mice **(Fig. 6C-D)**. As a consequence, the growth retardation by HRD1 ablation was largely rescued by AAV-shCrebh administration **(Fig. 6E)**. The growth hormone-JAK-STAT5 target genes, including *Igf1, Cyp2d9, cyp4a12,* were also rescued by AAV-shCrebh administration **(Fig. 6F)**. In addition, female infertility and estrus cycle of HRD1 LKO mice were largely rescued by AAV-shCrebh administration **(Fig. 6G-H)**. These results further confirming our conclusion that liver HRD1 executes its function partially through suppressing CREBH-mediated FGF21 production.

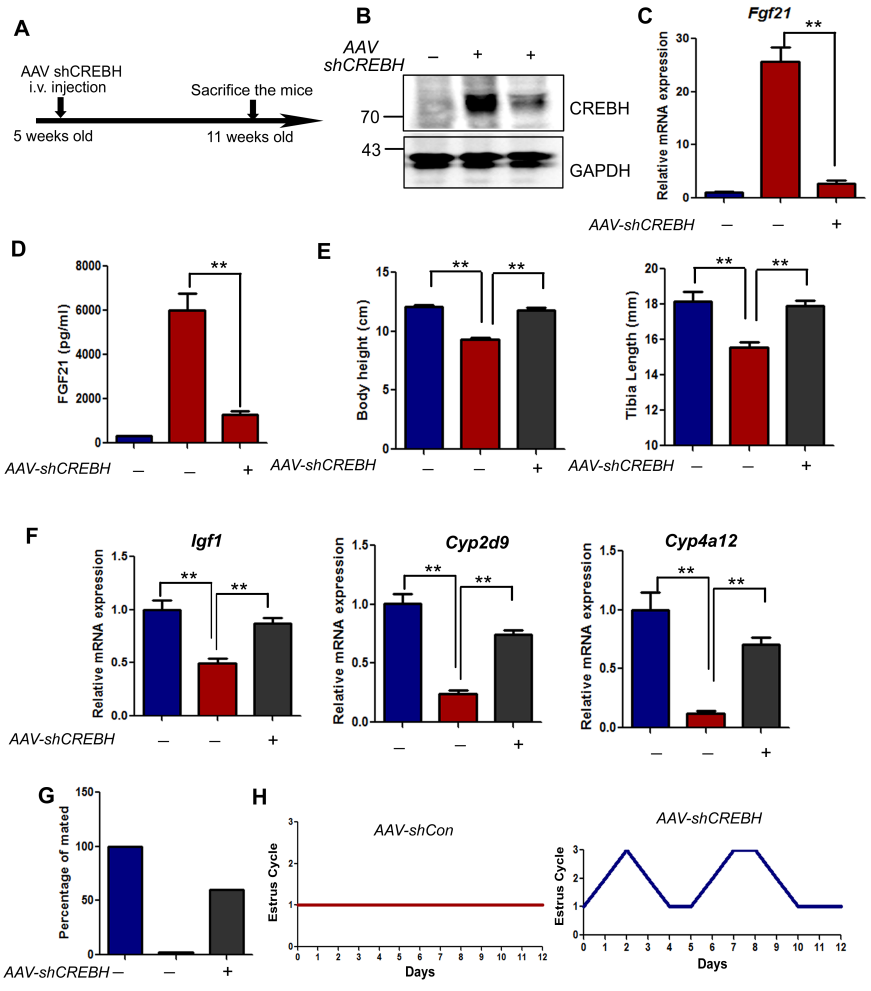


Fig. 6 CREBH ablation rescues the phenotypes induced by hepatic HRD1 deletion. (**A**) Flowchart of the study design for the knockdown CREBH in vivo. (**B**) Hepatic CREBH protein levels 5 weeks after AAV-sh*Crebh* injection. (**C**) Hepatic Fgf21 mRNA in the WT and L-HRD1 KO

mice 5 weeks after AAV-sh*Crebh* injection. (**D**) Serum FGF21 protein levels in the WT and L-HRD1 KO mice 5 weeks after AAV-sh*Crebh* injection. (**E**) Body height and tibia length of the WT and L-HRD1 KO mice 5 weeks after AAV-sh*Crebh* injection. (**F**) Hepatic *Igf1, Cyp2d9 and Cyp4a12* mRNA in the WT and L-HRD1 KO mice 5 weeks after AAV-sh*Crebh* injection. (**G-H**) Percentage of the mated and estrus cycle of the WT and L-HRD KO 5 weeks after AAV-sh*Crebh* injection. The data are representative of three independent experiments (mean ± s.d.). *: P<0.05. **: P<0.01 by unpaired student's t test.

2. The physiological relevance of the finding was not proven. The study showed an inverse correlation between CREBH and HRD1 during fasting-refeeding and good biochemical data to suggest a connection. However, it is unclear whether the increased HRD1 is the reason for reduced CREBH and FGF21 during refeeding. If this cannot be addressed experimentally, it should be commented on as a caveat to interpreting the results, and the title and abstract changed accordingly. In addition, PPARalpha is known as a major regulator of hepatic FGF21 transcription during fasting-refeeding, with CREBH as a potential cofactor. The relationship between CREBH and PPARa on the Fgf21 promoter in the absence or presence of ERAD as well as PPARa protein levels might be demonstrated to support the conclusions.

Reply: We initially showed in **Fig 2B & C** that both the hepatic *Fgf21* mRNA and circulating FGF21 were repressed in the refeeding condition in the WT mice, and this repression of FGF21 production is totally abolished by HRD1 deletion in liver, suggesting that HRD1 is responsible for refeeding-induced FGF21 reduction. We further showed that HRD1 upregulation by refeeding is positively associated with increased CREBH ubiquitination and reversely correlated with CREBH protein expression levels, and HRD1 deletion diminished these correlations. Nevertheless, we agree with the review that we do not have sufficient evidence to show that HRD1 is the only factor during this process; the data interpretation and discussion of the physiological function of HRD1 during fasting-refeeding have been revised as suggested.

As the reviewer pointed out, PPARα is known as a major regulator of hepatic FGF21 transcription. However, the binding of PPARα to FGF21 gene promoters in the livers of HRD1 LKO mice was comparable with WT littermate controls (**Fig. EV6A**). The hepatic mRNA and protein levels of PPARα were even decreased in the HRD1 LKO mice (**Fig. EV6B & C**), possibly due to a negative feedback by elevated FGF21. In contrast, our CHIP analysis a significant increase in CREBH binding to FGF21 gene promoters in the livers of HRD1 LKO mice (**Fig. EV6A**). Therefore, our data suggest that CREBH appears to be the dominant substrate of HRD1 in regulating FGF21 expression.

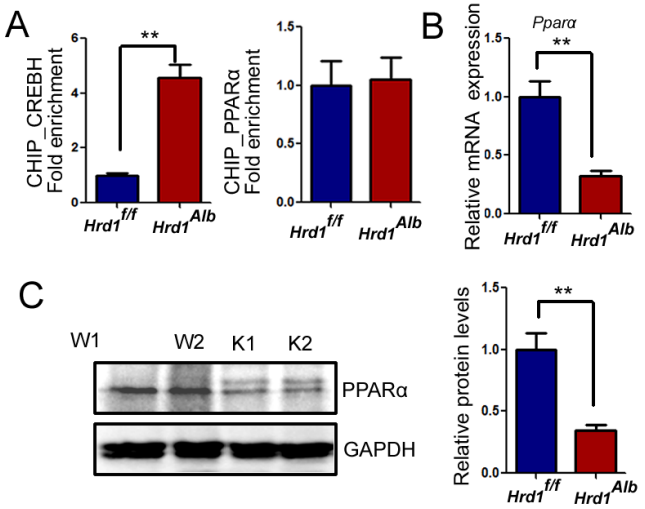


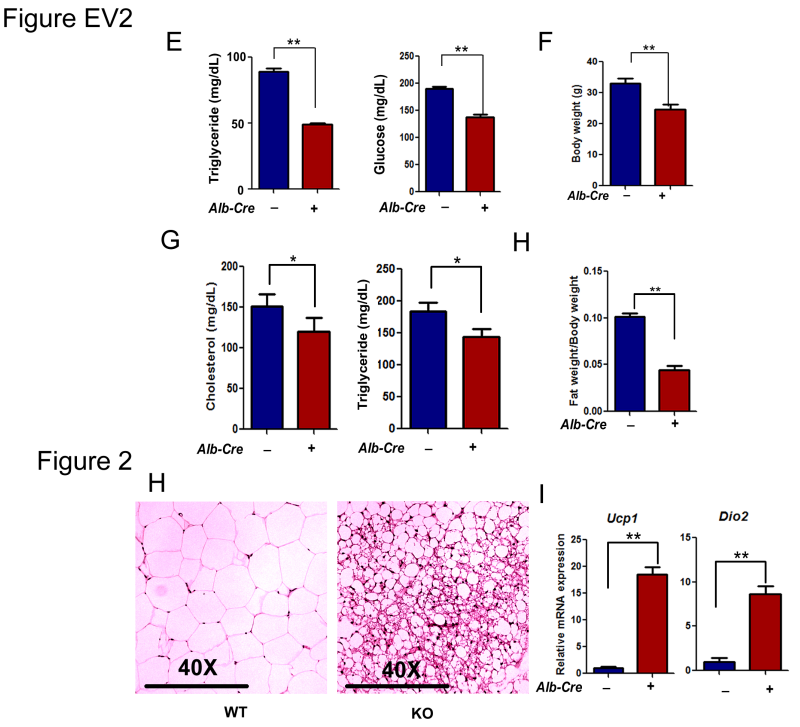
Fig. EV6. (A) Chromatin immunoprecipitation analysis of CREBH and PPARα binding onto the Fgf21 promoter in the livers of 12-week old mice, normalized first to 5% input group. (N=5 for each group). **(B)** Hepatic *Chop, Atf3* and *Psat1* mRNA levels in the WT and HRD1 LKO mice. (N=5 for each group). **(C)** The mRNA level of *Ppara*. (N=5 for each group). **(C)** Hepatic PPARα protein levels in the WT and HRD1 LKO mice. The data are representative of three independent experiments (mean ± s.d.). *: *P*<0.05. **: *P*<0.01 by unpaired student's t test.

3. The authors show that LKO mice exhibit some of the same phenotypes as FGF21 transgenic mice with respect to growth, activity and reproduction. Nevertheless, it would be more compelling to show these animals display some of the key beneficial effects of FGF21

overexpression, such as changes in blood glucose, triglycerides, ketone body levels, and resistance to diet induced obesity, etc. The authors should demonstrate at least of some of these other phenotypes.

Reply: We now provided new data showing that the blood glucose and TG levels were dramatically decreased in the HRD1 LKO mice **(Figure.EV2E)**. The body weight gain of HRD1 LKO mice was significantly less than that of the control mice **(Figure.EV2F)** 14 weeks after HFD feeding, indicating that HRD1 deletion protects mice from HFD-induced obesity. In addition, the accumulation of subcutaneous white adipose tissue and lipid levels were dramatically lower in HRD1 LKO mice compared to WT mice **(Figure. EV2G-H)**. Histological analysis demonstrated that the browning of the white adipose tissue in HRD1 LKO dramatically increased **(Fig. 2I)**. Moreover, mRNA of Mitochondrial uncoupling protein 1 (*Ucp1*) and Iodothyronine deiodinase 2 (*Dio2*) were also elevated in the white adipose tissue of HRD1 LKO mice (**Fig. 2J**). These results clearly indicate that, similar to FGF21 TG mice, liver-specific HRD1 deletion protects mice from HFD-induced obesity.

Fig. EV2E-H & 2I-J. Hepatic HRD1 ablation mice increase the browning of white adipose tissue. (**Fig. EV2E**) Serum TG and blood glucose of WT and HRD1 LKO mice under refed conditions. (n=6 for each group). (**B-D**) Body weights (**Fig. EV2F**), Cholesterol and TG (**Fig. EV2G**), Fat weight and body weight ratio (**Fig. EV2H**) of WT and HRD1 LKO mice 16 weeks after HFD feeding (n=6 for each group). (**Fig. 2H**) H&E stain of white adipose tissue WT and HRD1 LKO mice 14 weeks after HFD feeding. (**Fig. 2I**) *Ucp1* and *Dio2* mRNA levels of adipose tissue from (**E**). The data are representative of three independent experiments (mean ± s.d.). *: P<0.05. **: P<0.01 by unpaired student's t test.



4. The authors only showed that Hrd1 is sufficient to degrade CREBH, but not whether it is required for CREBH turnover. This is necessary to explain the elevated CREBH protein level in LKO liver. To this end, CREBH turnover should be examined in Hrd1 KO primary hepatocytes or cells.

Reply: We initially showed that HRD1 over expression fascinated CREBH degradation. To further determine CREBH turnover, primary hepatocytes were isolated from WT and HRD1 LKO mice. 24 hours after isolation, cells were treated with CHX for indicated time and the CREBH expression levels were determined. As expected, the half-life of CREBH protein dramatically increased in KO primary hepatocytes, further supporting our initial conclusion that HRD1 promotes CREBH degradation **(Fig. 4J)**.

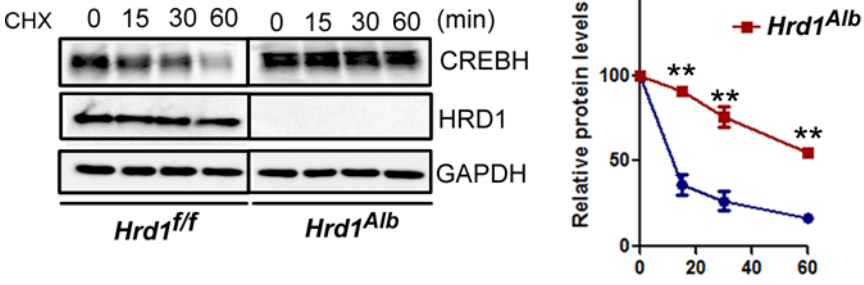


Fig. 4J. Primary hepatocytes were isolated from WT and HRD1 LKO mice and

Western Blot analysis CREBH protein stability.

5. It is confusing that the cytosolic domain of HRD1 is sufficient to degrade CREBH. The authors showed that only the ER-localized CREBH could interact with HRD1. As HRD1-C lacks the ER-transmembrane domain, how does it degrade CREBH on the ER? ***The intracellular localization of HRD1-C vs. full length should be compared, and whether HRD1-C degrades cleaved CREBH should be measured.*** *In line with this, the localization of CREBH in the ER and/or nucleus during fasting-feeding and in the LKO liver should be demonstrated.*

Reply: We show that HRD1-C, which lacks the ER-transmembrane domain, is sufficient to recognize the CREBH-FL (**Fig. 4L**) and increase its ubiquitination **(Fig. 5D & J).** We now provide new dada showing that HRD1-C largely localizes in the cytoplasm and partially co-localizes with the full-length CREBH (**Fig. EV7A),** suggesting that the HRD1-C binds to the cytoplasmic region of CREBH. As a positive control, a partial colocalization of the full-length HRD1 and CREBH are also confirmed (**Fig. EV7A**). In contrast, as reported before, the cleaved-CREBH are exclusively localizes in the nucleus of the cell (**Fig. EV7B**) and could not interact with HRD1-FL as showed in **Fig. 5B**. Accordingly, HRD1-C could not promote the degradation of cleaved-CREBH (**Fig. EV7D**).

Therefore, we propose that both CREBH-FL and CREBH-Activated were increased after HRD1 deletion as we expected. However, CREBH-FL is mainly localized in the cytoplasm and CREBH-activated is mainly localized in the nuclear.

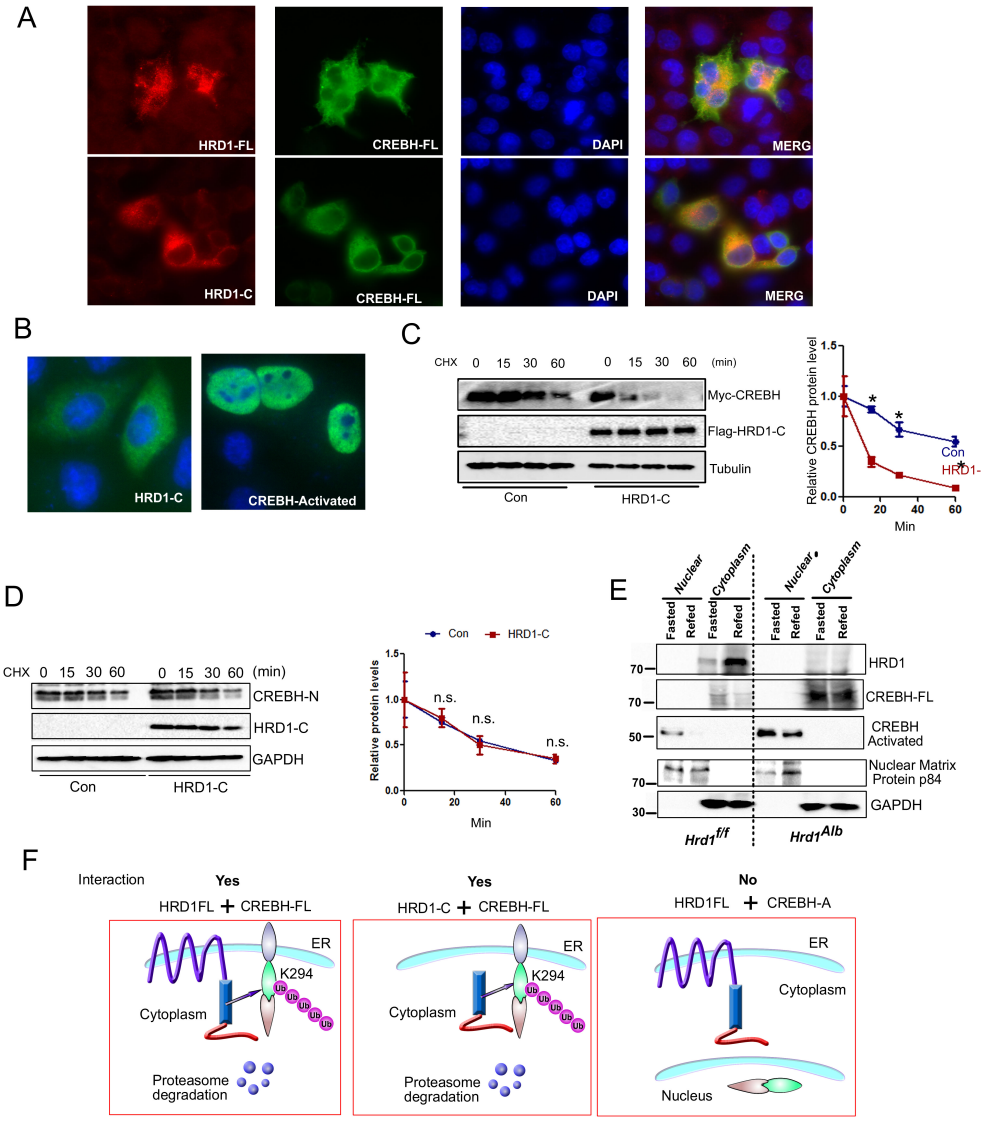


Fig. EV7. (A) Immunostaining of Flag-HRD1-C, Flag-HRD1 full-length and Myc-CREBH full-length 48 hours after transfection. **(B)** Immunostaining of Flag-CREBH-activated 48 hours after transfection. **(C-D)** Western Blot analysis cleaved form CREBH protein stability after HRD1-C terminal protein over-expression with CREBH full-length (**C**) CREBH-cleaved form (**D**). **(E)** Western blot analysis of hepatic HRD1 and CREBH of 16-weeks-old mice under overnight fasted or overnight fasted-refed 4 hours states; tissues were fractionated into nuclear and cytoplasm fractions. **(F)** A model of interaction between HRD1 and CREBH.

6. Using Hrd1/Fgf21 double knockout mice, the authors showed that FGF21 is responsible for the phenotypes of LKO mice. The authors should also provide some corollary data at the level of gene expression in the liver. The authors mentioned in the discussion session that the FGF21 deletion only partially rescued body weight loss in the LKO mice. These data should be shown to allow assessment on the data.

Reply: As suggested, we analyzed the expression of growth hormone signaling pathway target genes including *Cyp4a12, Cyp2d9* and *Hsd3b5*, all of which were partially rescued by FGF21 deletion (**Fig 7B & E).** These data further support our conclusion that HRD1 regulates systemic metabolism partially in a FGF21-dependent manner.

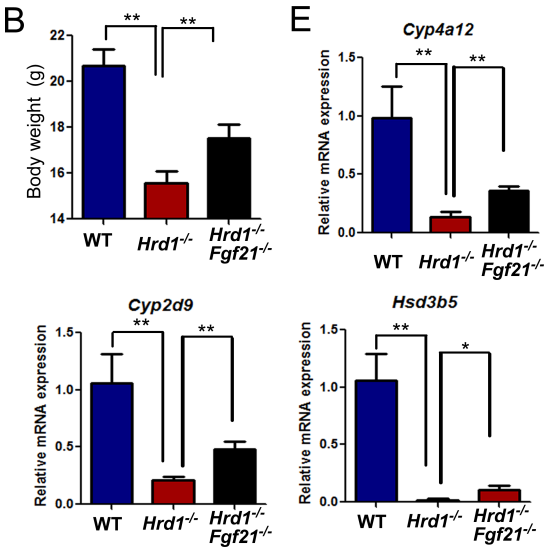


Fig. 7 Hepatic FGF21 ablation rescues the phenotypes induced by hepatic HRD1 deletion. (**B**) Body weight of the WT, L-HRD KO and HRD1/FGF21 DKO mice. (n=5 for each group). (**E**) Hepatic *Hsd3b5, Cyp2d9 and Cyp4a12* mRNA in the WT, L-HRD1 KO and HRD1/FGF21 DKO mice. (n=5 for each group).

Minor comments:

1. The authors observed the activation of PERK-ATF4 branch of UPR in the LKO liver. What happens to the other two branches of UPR? In fact, IRE1a-Xbp1 branch has also been shown to regulate FGF21 in the liver (PMID 25170079). The authors might explore this angle more in depth, although this is not necessary for this first report. In considering this, the effect of PERK inhibitor should be examined more carefully. ATF4 protein level and the expression of additional ATF4 target genes should be measured to demonstrate the inhibition of PERK-ATF4 activity.

Reply: We agree with the reviewer that it is important to analyze the activation of the IRE1a-Xbp1 branch and ATF4 branches in Hrd1-null liver, because both have been shown to regulate FGF21 expression in the liver. However, hepatic Xbp1s mRNA levels were comparable between WT and HRD1 KO mice, largely excluding the possibility that HRD1 regulates Fgf21 expression through the IRE1a-Xbp1 pathway (**Fig. EV5H**).

As suggested, we also examined the expression of ATF4 target genes, *Asns and Psat1,* to further evaluate the effect of PERK inhibitor. Our results showed that *Asns* and *Psat1* but not *Fgf21* expression were dramatically inhibited after PERK inhibitor administration (**Fig. EV5F**). We also showed that ATF4 protein level were decreased after PERK inhibitor administration (**Fig. EV5G**).

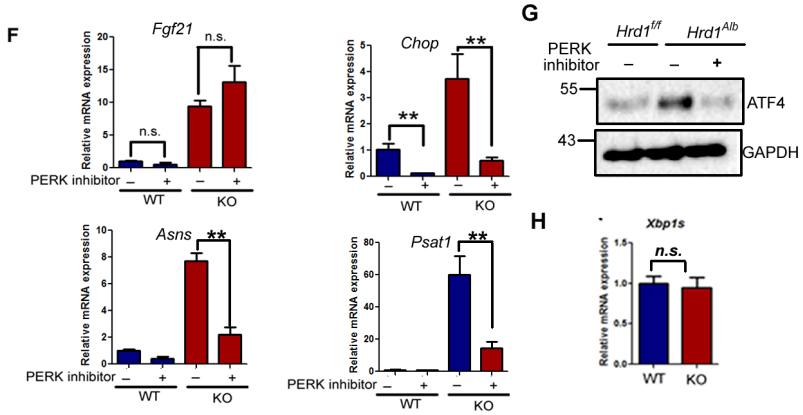


Fig. EV5. (F) *Fgf21* and *Chop*, *Asns* and *Psat1* mRNA expression after PERK inhibitor injection. (n=5 for each group). (**G**) ATF4 protein levels after PERK inhibitor injection. (n=5 for each group). (**H**) Xbp1s mRNA levels in WT and HRD1 KO mice. (n=5 for each group).

2. Sample size and number of repeats are not described throughout the manuscript. Statistical analyses are needed in Fig 4C, 4I, 4L and 5E.

Reply: The sample size and animal numbers are added to the Fig. legend through the manuscript. Statistical analyses are added in Figures 4C, 4I, 4L and 5E.

3. K27 ubiquitination is interesting and unexpected. Is it known whether this a CREBH- or Hrd1-specific phenomenon?

Reply: We agree with the reviewer that K27 ubiquitination is interesting and it is less known. It has been reported that E3 ligase RNF168 promotes K27 ubiquitination to signal DNA damage and endoplasmic reticulum (ER) localized E3 ligase AMFR increase K27 linked ubiquitination of STING after virus infection (Cell Rep. 2015 Jan 13;10(2):226-38; Immunity. 2014 Dec 18;41(6):919-33.)

4. In Fig 4H, unlike Fig 4D, the HRD1 and CREBH protein levels seems unaltered during fasting-refeeding. Please explain.

Reply: The original image was over exposed during signaling capture. The panel of HRD1 expression in a shorter exposure time is now used. Quantification by Image lab software from Bio-Rad show a significant increase in the expression levels of HRD in mouse liver after refeeding.

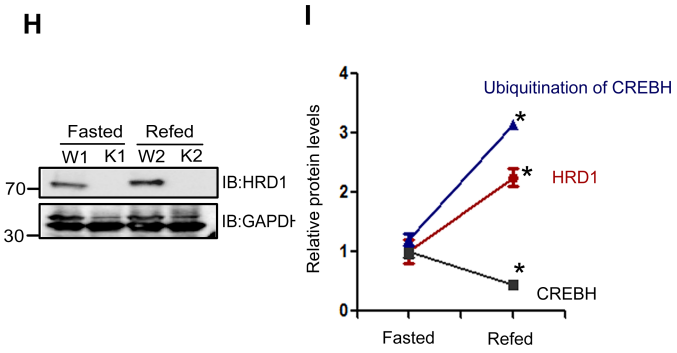


Fig. 4. HRD1 ERAD decreases the stability of CREBH through mediating its ubiquitination. (**H**) Hepatic CREBH ubiquitination level in the WT and L-HRD1 KO mice from **d**. (**I**) Relative protein levels of CREBH, HRD1 and ubiquitination of CREBH.

5. The endogenous co-IP in Fig 4F should include LKO liver as a negative control to avoid non-specific binding of CREBH to HRD1 antibody.

Reply: As suggested, HRD1-null liver tissue was used as a control to detect the interaction of HRD1 with CREBH. CRBEH protein was detected in anti-HRD1 immunoprecipitates from WT but not KO liver lysates.

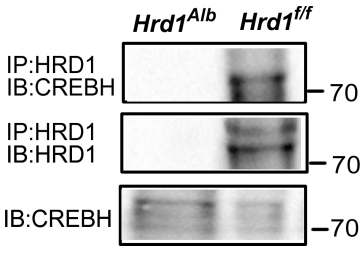


Fig. 4. (**F**) Endogenous interaction between CREBH and HRD1 in liver.

6. Some Fig. legends and panels were not cited correctly in the text. Please double check. There are two Fig 3 legends and no Fig 4 legend, and Fig 3H, S3E, 4G (CA mutant) were not mentioned in the text.

7. A thorough editing of English and grammar usage are needed.

Reply: Fig. citation was confirmed. A professional proof reading was used.

Referee #2:

In their manuscript "Hrd1-ERAD controls the hepatokine FGF21 production through K27-linked polyubiquitination of CREBH" Jucheng Wei et.al. address the metabolic function of the E3-ligase HRD1. Through an RNA sequencing experiment the authors identified FGF21 to be upregulated in a liver-specific

HRD1 knockout mouse (HRD1 LKO). The phenotypes of the HRD1 LKO mice phenocopy the FGF21 overexpression mouse line, such as female infertility, circadian behavior disruption and bone loss. Through a proteomics approach the authors further identified CREBH, which is a known transcription factor of FGF21, to be a target of HRD1. This finding was further verified by the authors through Co-Immunoprecipitation experiments and ubiquitylation assays. Additionally, they were able to show that only the full-length protein can interact with HRD1. Furthermore, they identified that CREBH is marked for degradation by an unusual K27-linked poly-ubiquitylation of the lysine 294 by HRD1. This missing regulation of CREBH in the liver-specific Hrd1 knockout mice leads to the upregulation of FGF21 which further leads to female infertility, circadian behavior disruption and bone loss. The phenotypes of the HRD1 LKO mice could be rescued by a knockdown of FGF21 by shRNA.

The data shown in this manuscript presents a metabolic function of the HRD1 E3-ligase.The data suggests a transcriptional regulation of FGF21 in the liver to control a crosstalk with multiple distal organs to control female fertility, circadian rhythm and growth. This work describes a so far uncharacterized role of HRD1, showing a new role of HRD1 in metabolic processes, which will definitely attract attention of the broad readership of EMBO *J.*

Major Comments:

• Fig 1A and S1A-B: in the text it is referred to that the authors identified Hrd1 in the proteomics experiment, however, Hrd1 is not listed in any of the named figures.

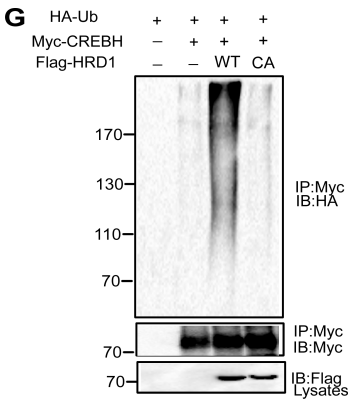
Reply: We apologize for not stated clearly. HRD1 was renamed as Syvn1 in 2005 due to its high expression in synovial cells from rheumatoid patients, which is now largely accepted as its official gene name. We now changed Syvn1 to Hrd1 to be consistent.

Fig. EV1. Generation of liver specific HRD1 knock-out mice. (A) ER stress target genes that are increased upon refeeding. (**B**) Hepatic mRNA profile in the fasting and refeeding condition.

• In the text the authors claim that they define a novel type of poly-ubiquitin chain for ERAD, however they never looked at other factors involved in ERAD. It could be that Hrd1 acts independent of the canonical ERAD pathway. This point is very important and needs further clarification.

Reply: We fully agree with the review that it is an extremely interesting (and important) question whether HRD1 ERAD executes its function exclusively through the canonical ERAD pathway. In fact, accumulated evidences suggests that HRD1 ERAD targets a group of transcription factors including p53, NRF1 & 2, BLIMP1, and the cell cycle suppresser $p27^{kip1}$ for degradation independent of ER stress response. In this case HRD1 recognizes these substrates through its C-terminal proline-rich domain but NOT its ER-lumen region. HRD1 target CREBH for degradation through its C-terminal proline-rich region (**Fig. 4L, 5A and Fig. EV7A-B).** This has been now discussed and our laboratory is currently further dissecting both the canonical ERAD-dependent and independent fashions of HRD1 at physiological and pathological settings, which we believe is beyond the scope of current study. We have discussed this interesting point in the revised manuscript.

• *Fig 4G: The Poly-ubiquitin Blot is not very convincing. The degree of polyubiquitination looks artificial? Maybe an additional full Myc-blot or Coomassie Gel would shed some light onto this poly-ubiquitination pattern.*



Reply: We repeated the experiment and data with better resolution are provided.

Fig. 4. HRD1 ERAD decrease the stability of CREBH through mediating its ubiquitination (G) Western Blot analysis ubiquitination of CREBH after immunoprecipitates of Flag-agarose 48h after transfection of Myc-CREBH, HA-Ub and Flag-HRD1 in HEK293T.

Minor Comments:

• *Fig 1A: The labeling is not clear, what is shown in lane 1 and what in lane 2?*

Reply: Thanks for the reviewer's comments, the labeling is added in **Fig 1A**.

• *Fig 4: Wrong label, is labeled as Fig 3.*
• *Fig 6B: There is a minus missing in the Blot labeling.*

Reply: All the blot labeling is carefully checked again.

• Fig 7: The model Fig. needs some more graphical clarity.

Reply: A model Fig. is added to the supplemental figures **(Fig. EV10).**

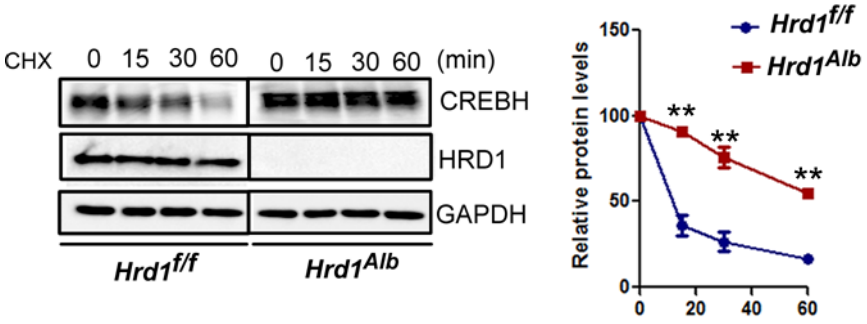
Referee #3:

In this manuscript, the authors describe the phenotypes of mice with conditional deletion of Hrd1 in the liver. These mice are smaller and the females are infertile. The phenotypes are similar to those of Fgf21 overexpressing mice. The authors then show that Fgf21 levels are increased and suggest that Hrd1 targets the degradation of Crebh, a ER-localized transcription factor for Fgf21. Finally, Fgf21 deletion rescues some of the phenotypes observed in mice with liver deletion of Hrd1.

Overall the study is interesting. It seems reasonable to postulate that Hrd1 targets Crebh since Hrd1 has been reported to target similar transcription factors in the same family including Oasis and Bbf2h7. However, direct demonstration of Crebh degradation by Hrd1 needs to be demonstrated. The authors rely on transfection of Hrd1 to show increased degradation of transfected Crebh. It will be more convincing to show that Crebh degradation is impaired in cells lacking Hrd1. This can be verified in hepatocytes or MEFs, for example. The requirement for Hrd1 in Crebh degradation should at least be demonstrated in cells with Hrd1 knockdown or knockout with appropriate reconstitution controls.

Reply: We agree with the reviewer that our original data were not sufficient to support the conclusion. As suggested, to further determine CREBH turnover, we isolated primary hepatocytes from WT and HRD1 LKO mice and compare CREBH protein stability in WT and HRD1-null primary hepatocytes. As showed in Fig. 4J, the half-life of CREBH protein dramatically increased in KO primary hepatocytes, further validating our initial conclusion that HRD1 promotes CREBH degradation in liver cells.

Fig. 4J. Primary hepatocytes were isolated from WT and HRD1 LKO mice and Western Blot analysis CREBH protein stability.



Crebh is one of many transcription factors regulating the expression of Fgf21. Although the authors are to be applauded for showing that deletion of Fgf21 rescues some of the phenotypes, it is not clear that increased Crebh is the sole reason for the increased Fgf21 in Hrd1 liver knockouts.

Reply: As what the reviewer pointed out that FGF21 expression is regulated by a group of transcription factors including PPARα, CREBH, ATF4 and Xbp1s. It is therefore important to elucidate whether HRD1 suppress FGF21 expression solely through CREBH degradation.

1. As shown in **Fig. EV6**, the binding of PPARα to Fgf21 gene promotor was comparable between WT and HRD1 LKO mice.

2. Pharmacological suppression of PERK, the upstream kinase for ATF4 transcriptional activation, while inhibited Chop transcription as expected, failed to suppress FGF21 expression (**Fig. EV5F**), largely excluding the possibility that HRD1 regulates FGF21 transcription through targeting ATF4.

3. Hepatic Xbp1s levels was also comparable between WT and HRD1 LKO mice as determined by qPCR (**Fig. EV5H**).

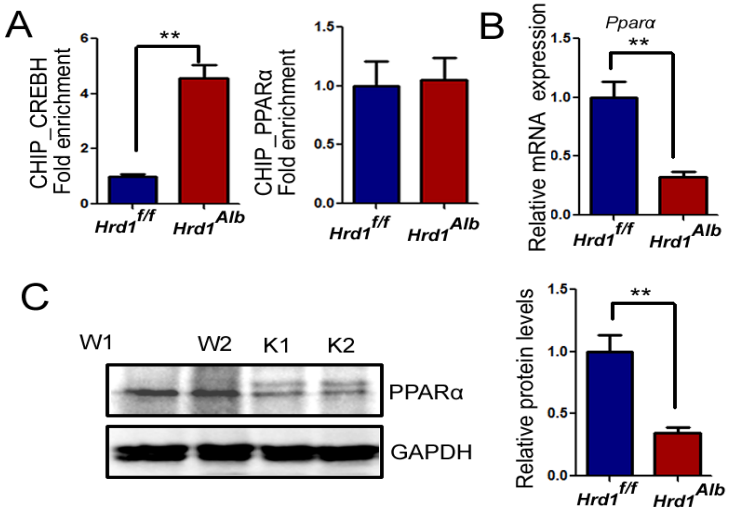


Fig. EV6. (A) Chromatin immunoprecipitation analysis of Crebh and PPARα binding onto the Fgf21 promoter in the livers of 12-weeksold mice, normalized first to 5% input group. (n=5 for each group). **(B)** Hepatic *Chop, Atf3* and *Psat1* mRNA levels in the WT and HRD1 LKO mice. (n=5 for each group). **(C)** Hepatic mRNA level of *Ppara*. (n=5 for each group). **(C)** Hepatic PPARα protein levels in the WT and HRD1 LKO mice. The data are representative of three independent experiments (mean ± s.d.). *: $P<0.05$. **: $P<0.01$ by unpaired student's t test.

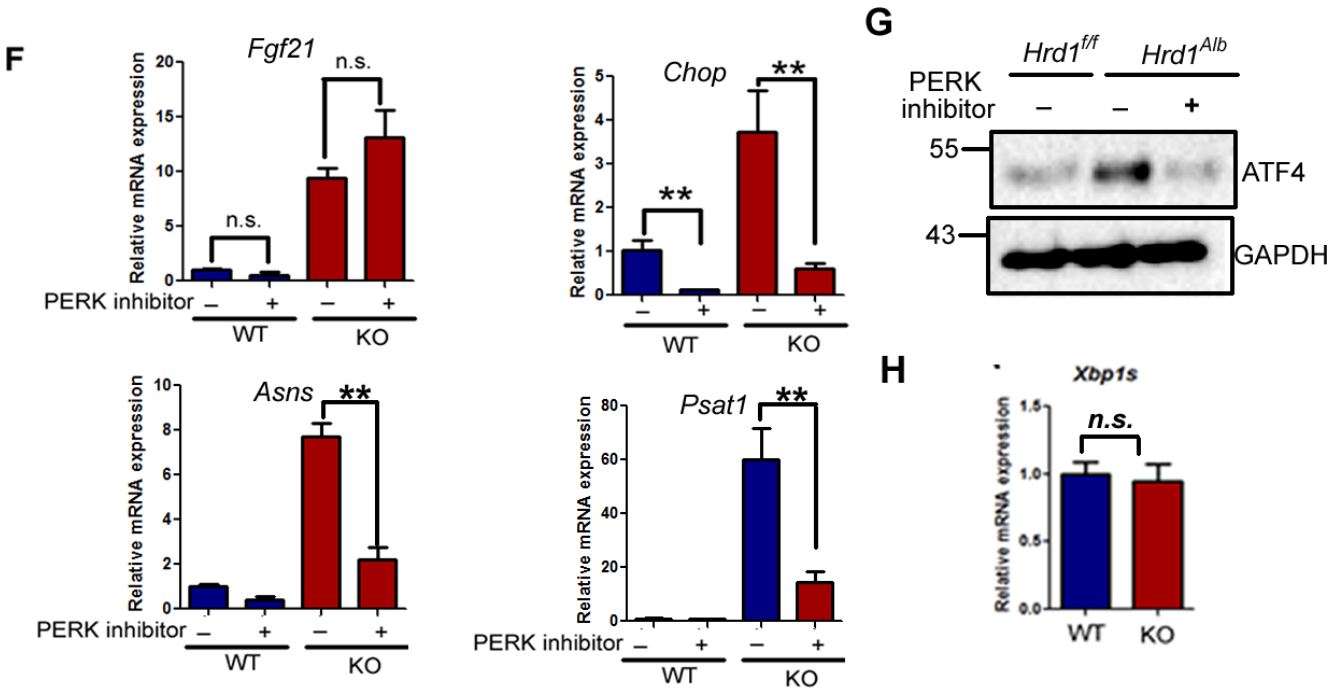


Fig. EV5. (F) *Fgf21* and *Chop*, *Asns* and *Psat1* mRNA expression after PERK inhibitor injection. (n=5 for each group). (**G**) ATF4 protein levels after PERK inhibitor injection. (n=5 for each group). (**H**) Xbp1s mRNA levels in WT and HRD1 KO mice. (n=5 for each group).

The authors try to emphasize the importance of K27 polyubiquitin chains in the regulation of Crebh. Since Crebh is presumbly degraded by Hrd1 ubiquitination, it is not clear what the significance of K27 linkage is in this model. However, if the authors want to establish a role for K27 ubiqutination, more data will have to be provided. Based on the model suggested by the authors, Crebh is ubiquitinated predominantly, if not exclusively, with K27 chains. In this case, single lysine mutants of ubiquitin will be dominant negative except for the mutant with K27 only. The data in Fig 5 seems to support this idea. However, there are variations, which probably reflect different expression levels of these mutants. This should be shown in the Fig. to allow assessment of the effects of these mutants on Crebh ubiquitination. More importantly, the authors show investigate the effects of expressing K27R mutant.

Reply: To exclude the variations, we measured the mutants of HA-Ub expression (**Fig. EV8A**). More importantly, we also showed that the increase of ubiquitination levels of CREBH by HRD1 only co-transfected by wild-type Ub but not K27R plasmid (**Fig. 5I & Fig. EV8B**).

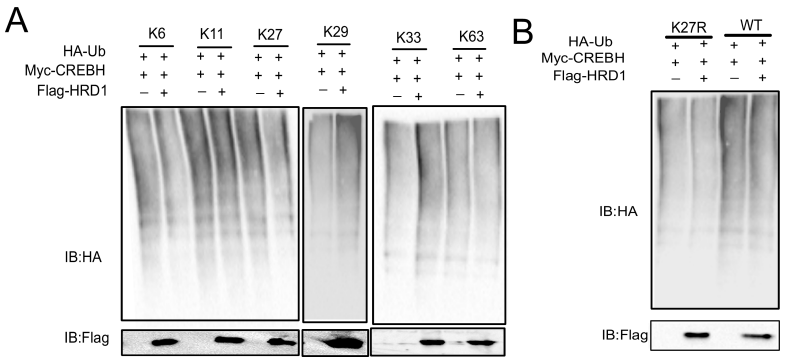


Figure. EV8. (A) Western Blot analysis of the CREBH K6, K11, K27, K29 and K33 only ubiquitination level after HRD1 co-expression. (**B**) Western Blot analysis of the CREBH WT, K27R ubiquitination level after HRD1 full-length co-expression.

If the proposed model is correct, this mutant should inhibit ubiquitination and degradation of Crebh in Hrd1 expressing or overexpressing cells. Similar experiments should be performed looking at ubiquitination of Crebh in single K to R mutants of Ub.

Reply: We agreed with the reviewer, if K27-poly ubiquitination is responsible for Crebh degradation, expression of K27R mutant of ubiquitin presumably functions in a dominant negative fashion to block Crebh degradation. However, given the fact that the endogenous ubiquitin is extremely abundant, together with the fact that Ub/K27R can still be used to form the chain end in K27R poly ubiquitination, it is often difficult to detect the dominant negative effect of a ubiquitin mutant.

Hrd1 can work with a number of E2s, none of which has been shown to make exclusively K27 chains. Is there another E3 involved?

Reply: We agreed with the reviewer that HRD1 can work with a number of E2s including UBE2J1, UBE2D2, UBE2D3 and UBE2G2. It has been showed that HRD1 interacted with UBE2D3 (UbcH5c) to increase the ubiquitination of APP (J Neurosci. 2010 Mar 17;30(11):3924-32) and HRD1 can also interact with UBE2D2 (UbcH5b) (Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14422-7) . Both UbcH5b and UbcH5c have been shown to make K27 chain to increase substrates ubiquitination (Cell Rep. 2015 Jan 13;10(2):226-38; Nat Commun. 2016 Jun 7;7:11792.).

The authors report that the cytoplasmic tail of Hrd1, without the transmembrane region, is enough to degrade Crebh. However, the transmembrane region of Hrd1 is required for Hrd1 interaction with Crebh. The authors should provide some insights into these observations. Will overexpressing another E3 achieve increase the degradation of Crebh?

Reply: There is a misunderstanding of our data. Our data showed that C-terminal but not the transmembrane of HRD1 is sufficient to interact with and induce the degradation of CREBH **(Fig. EV7F & Fig. 4 E, L, Fig. 5B).**

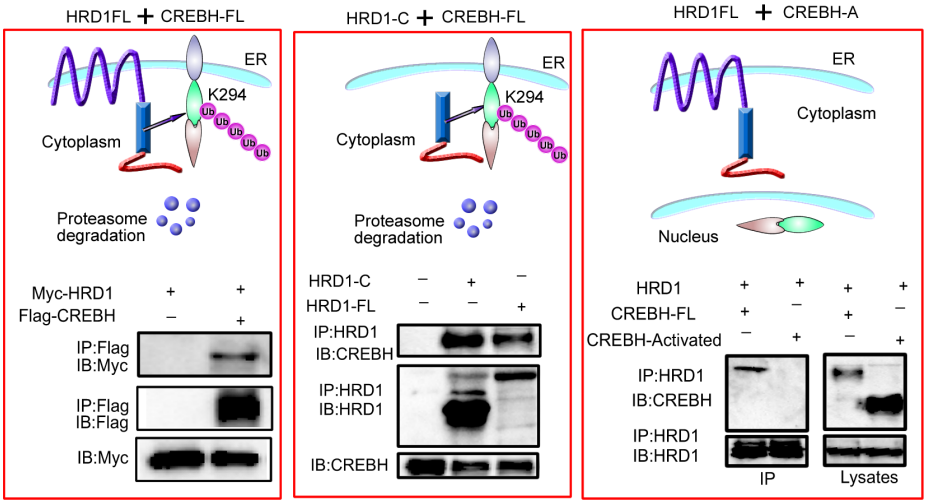


Fig. 4E, L and Fig. 5B. (**Fig. 4E**) Western Blot analysis interaction of CREBH and HRD1 after immunoprecipitates of Flag-agarose in transfected HEK293T. (**Fig. 4L**) Interactions of full-length and C-terminal of HRD1 with CREBH were measured by Co-IP. (**Fig. 5B**) Interactions of full-length and activated form of CREBH with HRD1 were measured by Co-IP.

Other comments:

The manuscript is poorly written. There is not enough information provided about the Methods used in this study. For example, the authors refer to an earlier paper describing the Hrd1 knockout mice but fail to provide a reference. It is important to describe the exons and domains of Hrd1 deleted in this model. In addition, it is important to confirm that no dominant negative truncation mutant is expressed. It is also not clear what cells are used in the different Fig. panels. The Fig. legends should provide enough information for readers to understand the experiments.

Reply: Thanks for reviewer's comment, we updated the Method information including:

1. New references about the HRD1 flox mice were added to **Method** section.

2. In, **Fig. EV1D**, we showed detail information to describe the exons and domains of HRD1 deleted in our model. And mRNA levels were dramatically decreased in HRD1 LKO mice.

3. The detail information in the Fig. legends was carefully revised to provide enough information.

The authors provide some description of their proteomics method. However, it is not clear if these are standard procedures or novel implementations. The authors claim that they have developed a method for measuring protein levels by spectral counting. However, without rigorous comparison to isotope labeling, it is not possible to know if their method is reliable. Simply showing the measurements are consistent between samples is not sufficient to guarantee the measurements are accurate. Variance can be reduced by data transformation, for example, such as log transform used in this study. In addition, the authors pad missing values with the lowest values of each replicate. What is the effect of this procedure on the statistics derived from the data?

Reply: It is a generic procedure for the proteomics method used in this manuscript. We have modified the manuscript as follows: "We used a highly reproducible label-free quantitative proteomic approach with an average Pearson correlation coefficient of 0.93" (Page 6, line 4). We measured the protein level through a label-free method by using the intensity (MS1) of proteins. We agree with the reviewer that the isotope labeling method is reliable to analyze the differentially expressed proteins. Label-free proteomics methods are also widely used in proteomics fields (Mol Cell Proteomics. 2014, 13(9): 2513–2526; Mol Cell Proteomics. 2013, 12(3):549-56; Proteomics. 2011, 11(4):535-53), which is also reliable to reach consistent conclusions. The consistence of replicated samples is an important indicator of the data reliability. As what the reviewer pointed out, due to the uneven identification of proteins by label-free proteomics method, a direct comparison to analyze the difference of proteomics data might lead to artifacts. Therefore, data transformation, i.e., to transform the raw proteomics data to the appropriate scale is essential to address potential artifacts for the data analysis (Mol Cell Proteomics. 2010, 9(12): 2704–2718). To analyze the variance by data transformation, we re-analyze the data using the original value (Intenstiy) of proteomics data. Consistence with original result, the average Pearson correlation coefficient of the biological replicates is 0.94, and the mean sample correlation coefficient with the logarithm of protein abundance was 0.93 (**Fig. EV3A**). Therefore, the log transform of proteomics data has little effect on the variance.

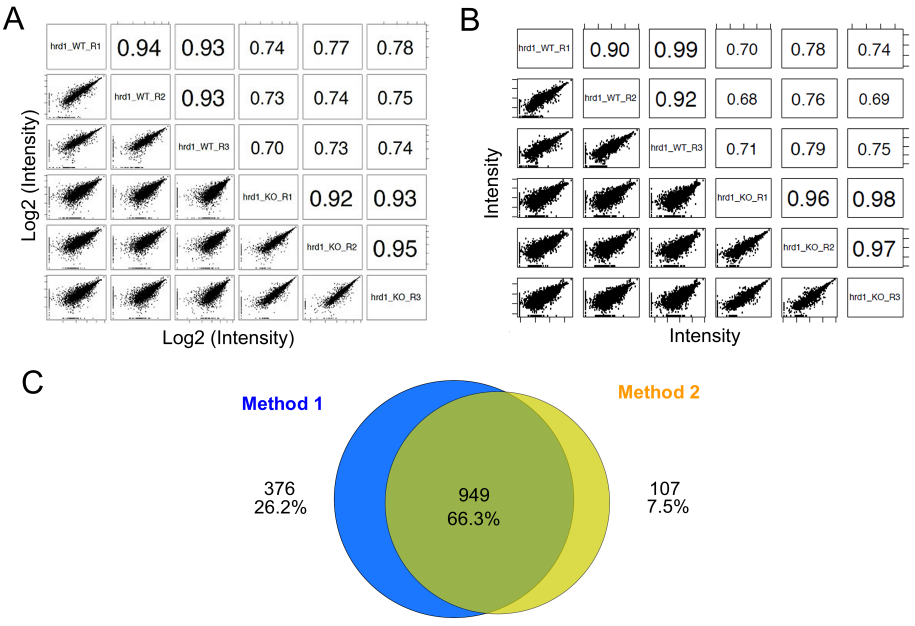


Figure EV3. Data analysis of the proteomic differential proteins between WT and HRD1 LKO mice. (**A**) Correlation analysis of proteomic data from WT and HRD1 LKO livers with logarithmic-transformed intensity of proteins. (**B**) Correlation analysis of proteomic data from WT and HRD1 LKO livers with original intensity of proteins. (**C**) Overlaps of the differentially expressed proteins between the missing values replaced with 1 (method 1) and replaced with minimum value of each replicate (method 2).

Due to the technical limits of proteomics, the same peptide is often observed only in a fraction of the samples, leading to the problems of missing values. To analyze the effect of the imputation procedure on the statistics derived from the data, we replaced the missing value with 1 (Due to the differential analysis cannot be conducted if the dataset contains some missing value) and re-analyzed the data. As indicated in the Fig. below, (Method 1: this reanalysis; Method 2: original data), the differentially expressed proteins were largely (66.3%) consistent for the two different imputation methods. When the missing value was used as 1, there are 26.2% more differentially expressed proteins were obtained compared with the Method 1 (replace missing values with 1). Due to the properties of missing values from proteomics data, it is more reasonable to use Method 2 than Method 1 in the manuscript. Thus, the major conclusion of this manuscript is unchanged.

Most of Figures 1, 2 and 3 belong to supplemental data.

Reply: We rearrange our figures.

Molecular weight markers should be shown throughout.
Reply: Molecular weight markers were added to Figures.

2nd Editorial Decision 25th July 2018

Thank you for submitting a revised version of your manuscript and please accept my apologies for the delay in coming back to you with a decision. Your study has now been seen by two of the original referees whose comments are shown below.

As you will see, referee #2 finds that all criticisms have been sufficiently addressed and recommends the manuscript for publication while referee #3 raises a few additional points that should be addressed in a final revision. The method section needs to be improved to better describe the lysis, IP and Co-IP conditions used. The referee also finds that further data is needed to support the physiological significance of K27 ubiquitin in your system. If you have further data on hand to address this issue then please include in the revised version. If not then please respond to this issue in the point-by-point response. Please also check/modify the text to make sure that you have a balanced discussion about this point and that you don't overstate the implications of the present data set. Please highlight text changes.

Also, there are a few editorial issues concerning text and figures that I need you to address before we can officially accept the manuscript.

REFEREE REPORTS

Referee #2:

The authors addressed all comments satisfactory and significantly improved the manuscript, which is a strong candidate for publication in EMBO J.

Referee #3:

The authors have greatly improved their manuscript and addressed most of my concerns. However, the methods are still not clearly written. For example, the lysis and IP conditions are not rovided. I checked the references cited but those papers also do not provide the conditions used. This is important for interpreting the results of the Co-Ip and IP experiments to examine crebh ubiquitination and must be provided to allow the readers to assess the reliabilty of the data.

One question that remains to be rigorously addressed is the physiological significance of K27 ubiquitin chain in this model. The authors show that expression of K27R reduces the ubiquitination of crebh when Hrd1 is overexpressed. This is based on assay by HA blotting, which detects the transfected Ub mutant. This is not ideal for showing the dominant negative effect of K27R Ub. If K27 is essential, its effects will be detected by one of two methods: Expression of Ub K27R will (2) inhibit crebh degradation and (2) inhibit polyubiquitin chain formation on crebh as detected by Ub blotting even when Ub K27R levels are low compared to endogenous Ub. The emphasis on K27 ubiquitination is premature and unjustified.

It is interesting that with ablation of crebh, Fgf21 levels remain substantially higher than wild type. This is further seen in the partial rescue of several genes in Fig 7. These should be pointed out in the statistical analysis in Fig 7.

Minor point:

Since Hrd1 C-terminus is in the cytosol, co-localization by fluorescence microscopy as presented is meaningless. Although activated crebh is in the nucleus, it will be relased by lysis. It is unexpected that full-length Hrd1 did not co-IP active crebh under these conditions while the cytosolic tail of Hrd1 did. This discrepancy should be addressed.

Referee #2:

The authors addressed all comments satisfactory and significantly improved the manuscript, which is a strong candidate for publication in EMBO J.

Reply: We thank the reviewer for the support.

Referee #3:

The authors have greatly improved their manuscript and addressed most of my concerns. However, the methods are still not clearly written. For example, the lysis and IP conditions are not rovided. I checked the references cited but those papers also do not provide the conditions used. This is important for interpreting the results of the Co-Ip and IP experiments to examine crebh ubiquitination and must be provided to allow the readers to assess the reliabilty of the data.

Reply: The experimental details are now provided.

One question that remains to be rigorously addressed is the physiological significance of K27 ubiquitin chain in this model. The authors show that expression of K27R reduces the ubiquitination of crebh when Hrd1 is overexpressed. This is based on assay by HA blotting, which detects the transfected Ub mutant. This is not ideal for showing the dominant negative effect of K27R Ub. If K27 is essential, its effects will be detected by one of two methods: Expression of Ub K27R will (2) inhibit crebh degradation and (2) inhibit polyubiquitin chain formation on crebh as detected by Ub blotting even when Ub K27R levels are low compared to endogenous Ub. The emphasis on K27 ubiquitination is premature and unjustified.

Reply: We agree with the reviewer that, if K27-poly ubiquitination is responsible for Crebh degradation, expression of K27R mutant of ubiquitin presumably functions in a dominant negative fashion to block Crebh degradation. As suggested, we have tried at least three times and saw a modest but rather not convincing reduction in CREBH ubiquination when K27R was over-expressed (lane 4 compare to lane 3). This is possibly because that the endogenous ubiquitin is extremely abundant, together with the fact that Ub/K27R can still be

used to form the chain end in K27R poly ubiquitination, it is often difficult to detect the dominant negative effect of a ubiquitin mutant.

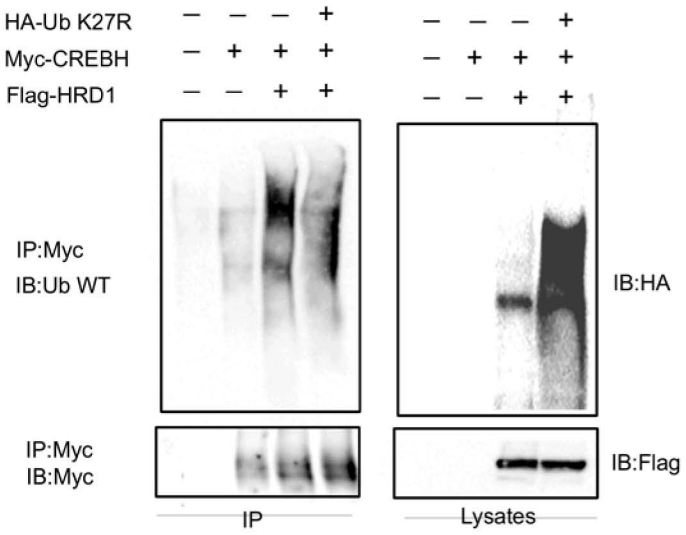


Figure: the effect of UbK27R expression on CREBH ubiquitination. Myc-CREBH, Flag-HRD1 and HA-Ub K27R expression plasmids were co-transfected into HEK293 cells as indicated. CREBH ubiquitination was determined by co-IP with anti-Myc and western blotting with anti-ubiquitin (left, top panel). The same membrane was reprobed with anti-CREBH (left, bottom panel). The levels of Ub K27R expression and Flag-HRD1 were determined by western as controls (right panels).

The results show that Hrd1 expression significantly enhanced CREBH ubiquitination (lane 3 compare to lane 2), which is consistent to our initial conclusion. K27R was over-expression resulted in a modest but rather not convincing reduction in CREBH ubiquination (lane 4 compare to lane 3). Therefore, this figure is not included in the manuscript.

It is interesting that with ablation of crebh, Fgf21 levels remain substantially higher than wild type. This is further seen in the partial rescue of several genes in Fig 7. These should be pointed out in the statistical analysis in Fig 7.

Reply: This is now addressed as suggested.

Minor point:

Since Hrd1 C-terminus is in the cytosol, co-localization by fluorescence microscopy as presented is meaningless. Although activated crebh is in the nucleus, it will be relased by lysis. It is unexpected that full-length Hrd1 did not co-IP active crebh under these conditions while the cytosolic tail of Hrd1 did. This discrepancy should be addressed.

Reply: We did not show any data of the interaction between Hrd1-C (the cytosolic tail of Hrd1) with the active CREBH. However, while lack of the ER localization, Hrd1-C interacts with the ER-resident transcription factor of CREBH possibly because the cytoplasmic region recruits the cytoplasmic Hrd1-C. Indeed, we detected a partial co-localization between the ER CREBH and the cytoplasmic Hrd1-C.

3rd Editorial Decision 3rd September 2018

Thank you for submitting a revised version of your manuscript addressing the remaining points raised by referee #3. I have looked at the manuscript and the point-by-point response and noticed that two issues pointed out by this referee are still not sufficiently addressed. I would therefore invite you to submit a final revised version of your manuscript in which you address the following points:

- Point 1 (lack of lysis and IP conditions): I noticed that one of the two references (Wei, Wei et al., 2014) does not report lysis and/or IP conditions neither in "Material and Methods" section nor in the "Supporting Information Material and Methods" sections. The second reference (Wei, Yuan et al., 2012a) describes lysis buffer composition only. Please replace Wei, Wei et al., 2014 with another reference providing a description of the biochemical procedures (or include the detailed conditions in the Materials and Methods section);
- Point 2 (physiological significance of K27 ubiquitination of CREBH in your model): I appreciate that you have aimed to address this point by testing the dominant negative effect of K27R ubiquitin overexpression on CREBH degradation. However, since the data provided remain inconclusive, I would kindly ask you to check/modify the text to make sure the conclusions of K27-linked CREBH ubiquitination are not overstated. I have proposed some text changes in the attached Word file (in track change mode) and I would ask you to review and approve them.

In addition, some editorial issues are still pending.

- Point 1 (lack of lysis and IP conditions): I noticed that one of the two references (Wei, Wei et al., 2014) does not report lysis and/or IP conditions neither in "Material and Methods" section nor in the "Supporting Information Material and Methods" sections. The second reference (Wei, Yuan et al., 2012a) describes lysis buffer composition only. Please replace Wei, Wei et al., 2014 with another reference providing a description of the biochemical procedures (or include the detailed conditions in the Materials and Methods section);

Answer: Wei, Wei et al., 2014 was now replaced with Xu et al, which provide a description of the detail biochemical procedures of co-IP and western blotting.

-Point 2 (physiological significance of K27 ubiquitination of CREBH in your model): I appreciate that you have aimed to address this point by testing the dominant negative effect of K27R ubiquitin overexpression on CREBH degradation. However, since the data provided remain inconclusive, I would kindly ask you to check/modify the text to make sure the conclusions of K27-linked CREBH ubiquitination are not overstated. I have proposed some text changes in the attached Word file (in track change mode) and I would ask you to review and approve them.

Answer: We agree with your concerns and have accepted all changes. All text and the supplemental file have been carefully checked and edited accordingly.

Accepted 17th September 2018

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Deyu Fang
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2018-98942

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We at least chose 5 samples for each group to make sure adequate power to decetc a pre-specified effect size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Statements about sample size were mentioned in Figure Legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Yes
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Yes
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA