Biochemical and Biophysical Research Communications 458 (2015) 34–39

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Depletion of the *cereblon* gene activates the unfolded protein response and protects cells from ER stress-induced cell death

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ARTICLE INFO

Article history:
Received 7 January 2015
Available online 22 January 2015

Keywords:
Cereblon
Endoplasmic reticulum stress (ER stress)
Unfolded protein response (UPR)
AMP-activated kinase (AMPK)
Stress response
Gene knockout

ABSTRACT

Previous studies showed that cereblon (CRBN) binds to various cellular target proteins, implying that CRBN regulates a wide range of cell responses. In this study, we found that deletion of the *Crbn* gene desensitized mouse embryonic fibroblast cells to various cell death-promoting stimuli, including endoplasmic reticulum stress inducers. Mechanistically, deletion of *Crbn* activates pathways involved in the unfolded protein response prior to ER stress induction. Loss of *Crbn* activated PKR-like ER kinase (PERK) with enhanced phosphorylation of eIF2α. Following ER stress induction, loss of *Crbn* delayed dephosphorylation of eIF2α, while reconstitution of *Crbn* reversed enhanced phosphorylation of PERK and eIF2α. Lastly, we found that activation of the PERK/eIF2α pathway following *Crbn* deletion is caused by activation of AMP-activated protein kinase (AMPK). We propose that CRBN plays a role in cellular stress signaling, including the unfolded protein response, by controlling the activity of AMPK.

1. Introduction

The Lon protease domain containing protein, *cereblon* (*CRBN*), was originally identified as a gene responsible for autosomal recessive non-syndromic mental retardation (ARNSMR) [1]. However, CRBN has since been characterized in various functional contexts [2–5] and increasing amount of evidences indicates that CRBN is involved in cell stress responses. For example, it has been reported to bind to the calcium-activated potassium channel (BK(Ca) channel) [2]. Binding of CRBN to thalidomide was determined to cause thalidomide-induced teratogenicity [3], and the binding of CRBN to immunomodulatory drugs has been characterized [6]. shRNA-mediated knockdown of CRBN induces cytotoxicity in myeloma cells, but the surviving cells showed strong resistance against immunomodulatory drugs [7]. Additionally, regulation of the α1 subunit of AMPK [4] and the β 4 subunit of the proteasome [5] by CRBN was recently identified. However, the physiological relevance and detail mechanisms of the role played by CRBN remain unclear.

The unfolded protein response (UPR) is a molecular mechanism by which cells maintain normal homeostasis in the endoplasmic reticulum [8]. The UPR triggers a series of signaling pathways that act to re-establish normal ER function under ER stress resulting in adaptation, alarming and cell death responses.

The adaptation mechanism of UPR enforces shutdown of global protein translation and induces the expression of selective genes to relieve stress. This is mediated by the activation of pathway that involves PKR-like ER kinase (PERK) and eukaryotic translation initiation factor 2 alpha (eIF2α). In stressed cells, active PERK inactivates eIF2α by phosphorylation. By the inactivation of eIF2α, global protein synthesis is slowed down and the flux of misfolded protein into the ER is limited. However, phosphorylation of eIF2α by PERK induces the selective translation of genes associated with

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http://dx.doi.org/10.1016/j.bbrc.2015.01.054
0006-291X/

stress-relieving machinery [9,10]. It has been well characterized that inactivation of eIF2α by prolonged serine phosphorylation can protect cells from ER stress-induced cell death [11–13].

If the adaptive UPR program fails to restore normal homeostasis in cells, then this program eliminates damaged cells by activating the apoptotic pathway. This implies that there are mechanisms that are able to functionally assess and respond to the strength level of stress [14].

To examine the role of CRBN in cellular stress signaling, we constructed a knockout mice strain in which *Crbn* was deleted throughout the body [15]. Here, we show that mouse embryonic fibroblast cells from *Crbn*-deficient mice are strongly resistant to endoplasmic reticulum stress-mediated cell death, and that loss of *Crbn* induces preactivation of the UPR, including activation of the Ire1 and PERK/eIF2α pathways.

2. Materials and methods

2.1. Experimental animals

Mice were housed within pathogen-free condition, and all experiments were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee.

2.2. Induction of hypoxia/reoxygenation

Induction of hypoxia was accomplished using the GasPak™ Plus Anaerobic System (BBL, BD Biosciences, Franklin Lakes, USA). In brief, cells were grown in DMEM under normoxic conditions, washed, and incubated in DMEM without FBS for 3 h. Cells were then further incubated in DMEM without FBS either at 37 °C in a humidified atmosphere of 5% CO_2 or in a chamber containing a GasPak for 46 h. Cells exposed to hypoxia were subsequently reoxygenated for 5 h.

2.3. Cell viability analysis

Cell survival was determined with the EZ-Cytox Cell Viability Assay Kit (Daeil Lab Service, Korea) by the manufacturer's protocol.

2.4. Histological analysis of the kidney

Male mice (8-weeks-old) were injected intraperitoneally with tunicamycin (3 μg/g body weight). After 3 days, mice were sacrificed and kidneys were fixed in 10% formalin and embedded in paraffin. Paraffin sections (5 μm) were then stained with hematoxylin and eosin.

2.5. Focal cerebral ischemia

Focal cerebral ischemia was performed using 9 week-old male mice (n = 16 for WT mice and n = 15 for KO mice). Ischemia was induced by occluding the MCA, as described previously [16,17]. Size of the cerebral infarct was determined on 2,3,5-triphenyltetrazolium chloride (TTC)-stained, 2 mm-thick brain section. Infarction area was quantified using iSolution full image analysis software (Image & Microscope Technology, Vancouver, Canada). Volume of infarction was quantified by indirect measurement using the sum of the volumes of each section by the following calculation: contralateral hemisphere (mm^3) − ipsilateral undamaged hemisphere (mm^3).

2.6. Immunoblot analysis

Cell extracts were prepared by adding lysis buffer (50 mM Tris-Cl, pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 2.5 mM β-glycerophosphate and protease inhibitor mixture from Roche Applied Science) to collected cells. After protein concentration determination, 30 μg of cell extract was analyzed by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting using polyvinylidene difluoride membranes (Bio-Rad). Proteins were detected by Super Signal West Pico chemiluminescence substrate (Pierce) with exposure to x-ray film.

3. Results

3.1. Crbn KO MEF cells are resistant to low oxygen stress-induced cell death in vitro and in vivo

We previously described the generation of *Crbn* KO mice to elucidate the function of CRBN [15]. Embryonic fibroblast cell lines established from the embryos of wild-type and *Crbn*-deficient mice were subjected to hypoxia-reoxygenation culture conditions to examine their response. While wild-type MEF cells undergo massive cell death, *Crbn*-deficient MEF cells were resistant to cell death, as confirmed both visually and by trypan blue exclusion assay (Fig. 1A and B). The molecular mechanism of resistance to cell death was again examined by immunoblotting for caspase-3 (Fig. 1C). To test the effect of *Crbn* loss on the cellular response to low oxygen-induced stress *in vivo*, wild-type and *Crbn* KO mice were subjected to cerebral ischemic injury. As Fig. 1D and E shows, brain from mice with CRBN deficiency got smaller infarct size comparing with wild type mice brain.

3.2. Crbn KO MEF cells are resistant to various types of stress-induced cell death

Aforementioned results raised the question about the specificity of cell death signaling involving CRBN. To address this, cells were challenged with a variety of cell death-inducing agents, including staurosporine (a pan kinase inhibitor), etoposide (a DNA damaging agent), paclitaxel (a mitotic destabilizing agent), tumor necrosis factor α with cycloheximide (a combination inducing extrinsic cell death pathway), thapsigargin (a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor), tunicamycin (an *N*-linked glycosylation inhibitor), brefeldin A (a Golgi complex transport inhibitor), and hydrogen peroxide (a reactive oxygen species producer). As shown in Fig. 2A, *Crbn* KO MEF cells are strongly resistant to cell death induced by etoposide, hydrogen peroxide, and the ER stress inducers tunicamycin, thapsigargin, and brefeldin A. However, *Crbn* KO MEF cells did not show any difference in cell survival following treatment with staurosporine or the TNF/cycloheximide combination. With regard to the microtubule targeting agent paclitaxel, *Crbn* KO MEF cells were resistant within a minimal range. This result indicates that CRBN is involved in specific cellular responses to stress.

When the test agents were classified by the type of stress, we observed that *Crbn* KO MEF cells were strongly resistant to cell death induced by ER stress revealed by survival assay (Fig. 2A left graph), pictograph (Fig 2A right panel), and caspase activation (Fig. 2B). GRP78, a marker of ER stress, was upregulated by tunicamycin in all tested MEF cells (Fig. 2B), implying that upstream UPR events were not affected by *Crbn* deletion.

We next confirmed resistance to cell death conferred by *Crbn* knockout by examining ER stress-induced kidney damage *in vivo*. Intraperitoneal injection of tunicamycin induced kidney damage in wild-type mice, while the kidneys of *Crbn* KO mice were resistant to damage. Gross inspection of the kidney sections which were stained with hematoxylin and eosin from tunicamycin-injected *Crbn* knockout mice showed less zonal drop-out of epithelial cells compared with wild type mice (Fig. 2C). Taken together, these data suggest that CRBN may be an essential component of apoptotic signaling in ER stress-induced cell death.

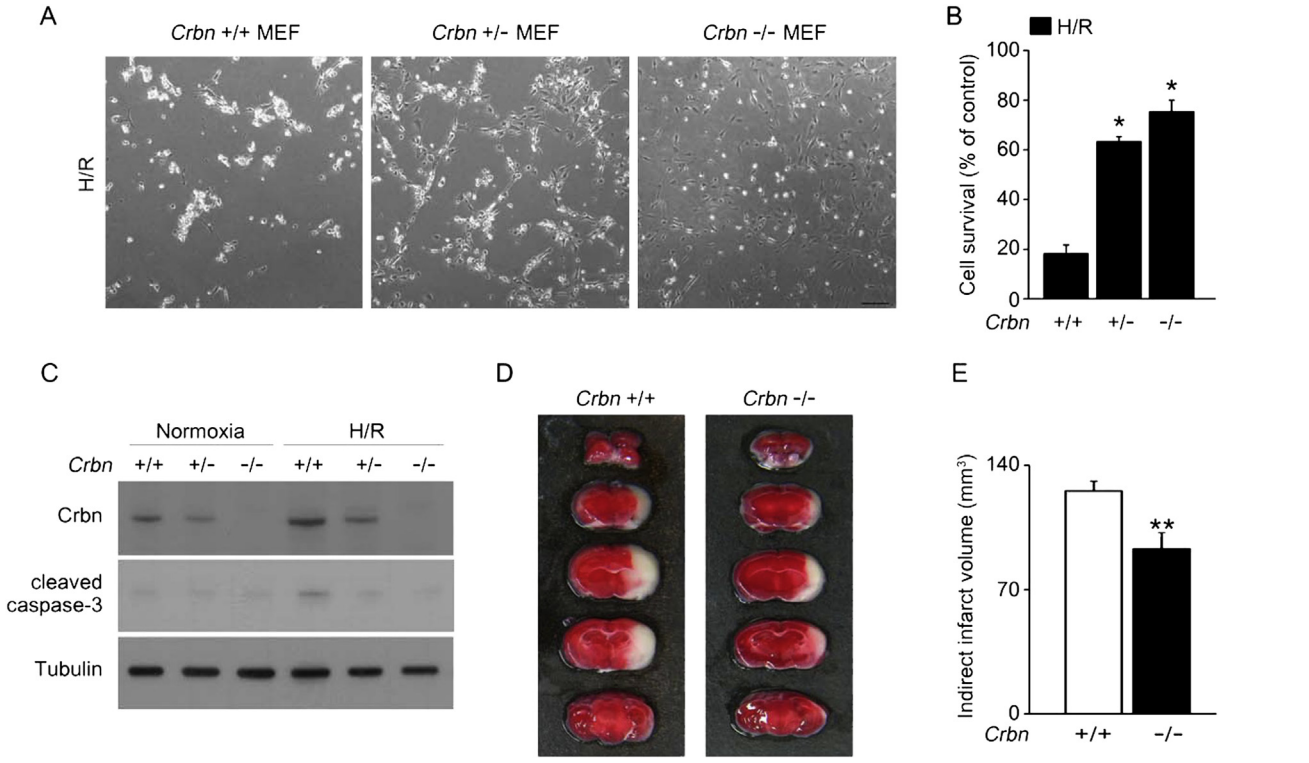

Fig. 1. *Crbn* KO MEF cells are resistant to low oxygen stress-induced cell death. (A) Images of MEFs (*Crbn* +/+, *Crbn* +/−, and *Crbn* −/−) under hypoxia − reoxygenation stress. (B) Relative cell survival rates were presented using trypan blue staining of cells from (A). Data represent mean ± SEM. $^{*}P < 0.001$ versus *Crbn* +/+ ($n = 4$). (C) Western blots of endogenous Crbn and cleaved caspase-3 in MEFs under either normoxia or hypoxia/reoxygenation (H/R). The results shown are representative of four independent experiments. (D) Images of coronal brain sections after focal cerebral ischemic injury stained with TTC. Red staining indicates healthy tissue and the white indicates infarcted tissue. (E) Quantification of indirect infarct volume in (D). Data represent mean ± SEM. $^{**}P < 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. UPR is pre-activated in Crbn KO MEF cells

To investigate the mechanism by which *Crbn* KO MEF cells are resistant to ER stress, we examined activation of the UPR in each MEF cell line. As shown in Fig. 3A, we confirmed that the signaling pathways of the UPR were pre-activated before ER stress induction. For example, PERK was activated in *Crbn* hetero- and homo-deficient MEF cells and, accordingly, the phosphorylation of eIF2α was also enhanced. This led us to investigate the activation of other UPR proteins, including GRP78, the most upstream marker protein of UPR, and p38 mitogen activated protein kinase (p38 MAPK), a downstream component of the Ire1 pathway. As shown in Fig. 3A, the expression of GRP78 was not modulated in *Crbn* KO MEF cells, and p38 MAPK was activated in *Crbn* KO MEF cells.

Next, we investigated whether *Crbn* depletion affects the biochemical activity of eIF2α regulation in response to ER stress in MEF cells with a time-dependent manner (Fig. 3B). In wild-type MEF cells, eIF2α was phosphorylated within 1 h of ER stress induction, and dephosphorylation was evident at later time points (Fig. 3B). However, the dynamics of eIF2α phosphorylation were different in *Crbn* KO MEF cells. In accordance with Fig. 3A, phosphorylation of eIF2α was enhanced before ER stress induction in *Crbn* KO MEF cells, and the intensity of eIF2α phosphorylation was not noticeably changed after ER stress induction (Fig. 3B).

3.4. AMPK is associated with activation of PERK in Crbn KO MEF cells

To confirm whether CRBN affects PERK and eIF2α phosphorylation status, the effect of Crbn overexpression on the PERK/eIF2α pathway was examined. As shown in Fig. 4A, reconstitution of Crbn in *Crbn* KO MEF cells reduced phosphorylation of PERK and, accordingly, phosphorylation of eIF2α was diminished. These results suggest that CRBN is involved in the regulation of PERK-eIF2α activity.

As we reported previously, CRBN inhibits the activity of AMPK *in vitro* by interacting with the α1 subunit of AMPK (AMPK α1) [4], and activation of AMPK was reported to play a protective role against ER stress-associated disruption of cell homeostasis [18–23]. We tested whether constitutively active (CA) and dominant-negative (DN) forms of AMPK would affect activation of the PERK pathway. As shown in Fig. 4B, forced expression of the CA form of AMPK enhanced eIF2α phosphorylation in wild-type MEF cells, while expression of the DN form of AMPK in *Crbn* KO MEF cells resulted in reduced phosphorylation of PERK and eIF2α. These data imply that activation of AMPK by CRBN depletion led to activation of the PERK- eIF2α axis of the UPR.

4. Discussion

CRBN has been implicated in control of various cellular processes raising the possibility that it plays a role in various cellular stress responses [4,5,15].

Although MEF cells lacking Crbn expression were strongly resistant to various types of cell death induction (Figs. 1 and 2), cumulative information about CRBN and its related signaling including AMPK and NRF2, prompted us to investigate the role of CRBN in ER stress-induced cell death [24–30]. From our data, it was evident that cell death by ER stress was significantly suppressed in *Crbn* KO MEF cells. This results was further confirmed in an animal model of ER stress-induced toxicity in kidney (Fig. 2C). Thus, our

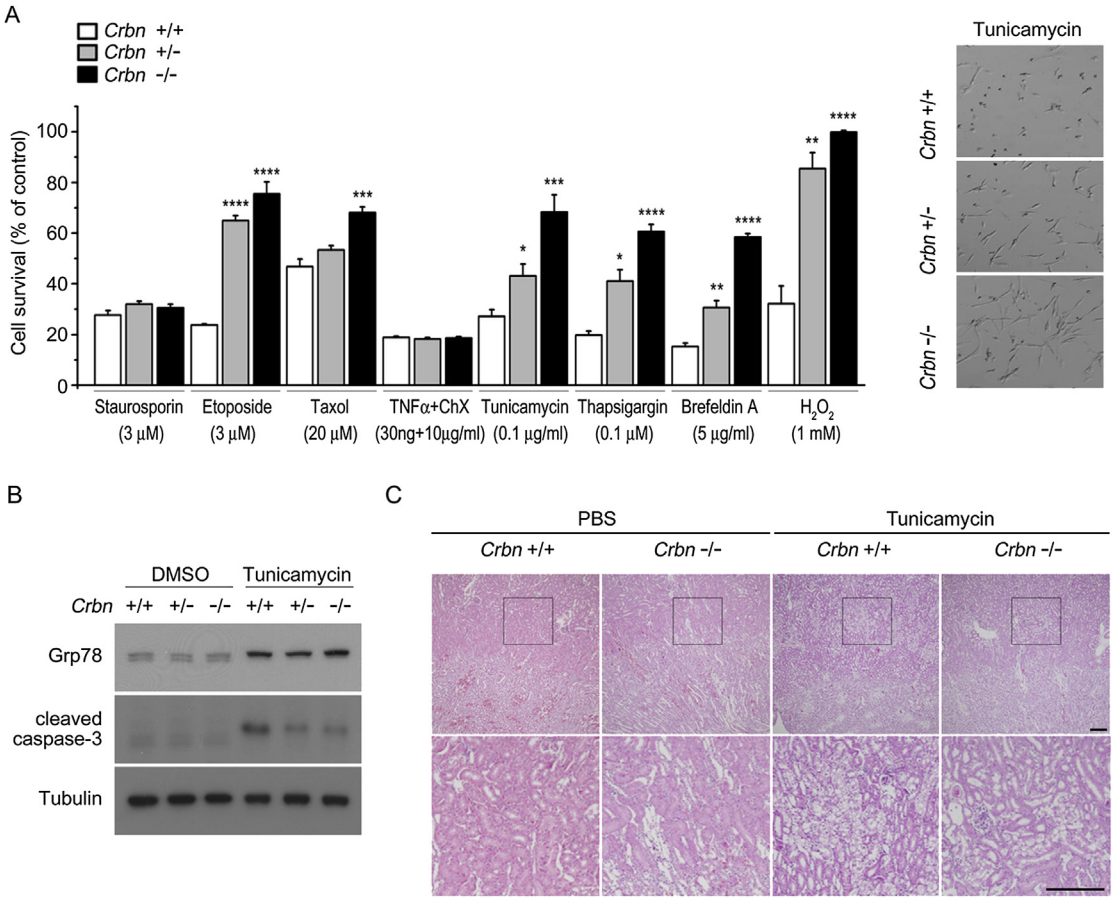

Fig. 2. *Crbn* KO MEF cells are resistant to ER stress-induced cell death. (A-left graph) MEFs were incubated with the indicated reagents for 24 h and then analyzed for cell survival. Data represent mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$ versus *Crbn* +/+. (A-right panel) Images of MEFs incubated with 5 μg/ml tunicamycin for 24 h. (B) Western blots of endogenous Grp78 and cleaved caspase-3 in MEFs treated with either DMSO or tunicamycin (1 μg/ml). (C) WT and *Crbn*-KO adult mice were injected intraperitoneally with 3 μg/g tunicamycin. Kidney sections were stained with hematoxylin and eosin. The regions in the upper images outlined in black are shown in the bottom panels. Scale bar = 20 μm.

data support a role for CRBN in mediating ER stress-induced cell death. Probably, CRBN is not required for the regulation of cell death developmental program, as *Crbn*-deficient mice are apparently normal Ref. [15]. Instead, our results imply that CRBN functions in a pathological view with expression regulation, when the cells are facing stress-inducing conditions.

Mechanism of resistance to cell death in *Crbn* KO MEF cells is characterized by preactivation of the UPR, including AMPK-

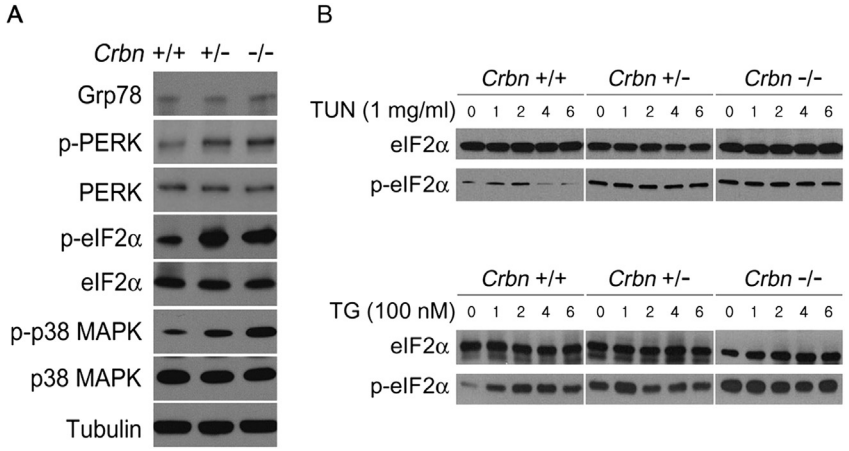

Fig. 3. Activation of the PERK-eIF2α pathway in *Crbn* KO MEF cells. (A) Extracts of MEF cells were prepared as described in Materials and Methods. The lysates were probed with the indicated antibodies. The results shown are representative of two independent experiments. (B) MEFs were treated with tunicamycin (1 μg/ml) or thapsigargin (100 nM) for the indicated times. The prepared lysates were probed with anti-phospho-eIF2α or anti-eIF2α antibody. The results shown are representative of two independent experiments.

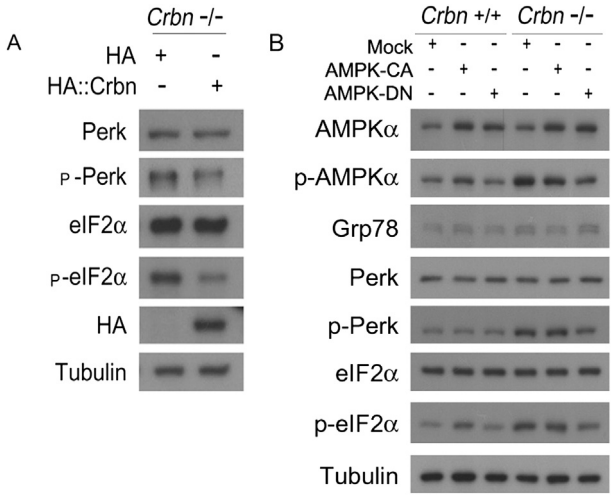

Fig. 4. Activated AMPK in *Crbn* KO MEFs triggers activation of the unfolded protein response. (A) *Crbn* −/− MEFs were transiently transfected with either HA:CRBN or an empty HA vector. Cell lysates were probed with anti-PERK, anti-phospho-PERK, anti-eIF2α, anti-phospho-eIF2α, or anti-HA. The results shown are representative of four independent experiments. (B) *Crbn* +/+ and *Crbn* −/− MEFs were transiently transfected with empty vector (Mock), AMPK-CA, or AMPK-DN. Cells were harvested after 24 h. The lysates were probed with the indicated antibodies. The results shown are representative of four independent experiments.

dependent phosphorylation of eIF2α. As well characterized already, phosphorylation of eIF2α at Ser51 inhibits global translation of mRNA reducing protein neogenesis suggesting that the reduced protein load into ER causes the resistance of *Crbn* KO MEF cells to ER stress-induced cell death. This hypothesis was confirmed recently by showing the reduced protein translation rates in *Crbn* KO MEF cells [31].

We also considered the possible involvement of XBP1 protein in the resistance of *Crbn* KO MEF cells [9,32]. However, our additional investigation about XBP1 splicing did not show any impressive changes in *Crbn* KO MEF cells (data not shown) implying that role of CRBN in UPR is limited in PERK pathway.

As we reported previously, AMPK activity was significantly enhanced in *Crbn* -deficient cells and animals [4,15], and a protective role for AMPK against ER stress has been repeatedly suggested. Interestingly, studies using AMPK activators strongly support our results. Metformin, an AMPK activator, played a cytoprotective role against ER stress-induced cell death in mouse pancreatic beta cells [29]. In this regard, our data is comparable with other reports, and our results suggest that CRBN is a molecular switch that regulates the AMPK-UPR signaling network in response to ER stress.

Although the function of CRBN in regulating the UPR (PERK, Ire1) is addressed here, some basic questions are still not answered. First, it is not clear whether the resistance phenotype of *Crbn*-deficient MEF cells is caused only by preactivation of the UPR. Second, cytoprotection conferred by AMPK activity via the UPR raised the possibility of an unknown molecular signaling network between AMPK and UPR. And lastly, it will be important to examine whether the loss of *Crbn*-dependent UPR regulation via AMPK is conserved in humans.

In summary, this study provides the first *in vivo* and *in vitro* evidences that CRBN is involved in the activation of PERK and the Ire1 pathway of the UPR, and that CRBN deficiency protects cells from ER stress-induced cell death. Taken together, our results suggest that CRBN can be considered a novel regulator of protein translation-associated stress response, and that CRBN is a possible therapeutic target for the treatment of human disorders related to ER stress.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was funded by the 'General research grant of Asan Institute for Life Sciences [2014-512]' to IKK, and by grants for the National Leading Research Laboratories [2011-0028665] and the Science Research Center of Excellence Program [2007-0056157] of National Research Foundation of Korea and the Translational Research Grant [H13C1412] of Korea Health Industry Development Institute of Korea to CSP.

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