Cereblon induced mitochondrial dysfunction ameliorates drug resistance of multiple myeloma

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Abstract:
Background: Cereblon (CRBN) is a thalidomide binding protein which plays an essential role in anti-myeloma effects associated with the teratogenicity of immunomodulatory drugs (IMiDs). CRBN expression level may serve as a potential biomarker to predict patients' response to IMiDs. However, CRBN related mitochondrial biomarkers in MM have not been reported.
Objective: To identify the correlation between CRBN and mitochondrial function modification in MM.
Methods: To identify the alteration of CRBN and mitochondria, we performed western blotting, quantitative real-time polymerase chain reaction, gene transfection, adenovirus infection and immunocytochemistry. For the mitochondrial functional analysis, the mitochondrial membrane potential, reactive oxygen spaces, mitochondrial mass and mitochondrial ATP level were measured by laser confocal microscopy and flow cytometry.
Results: CRBN expression depends on drug resistance and susceptibility of MM cells. Intriguingly, CRBN negatively regulates mitochondrial function. Mitochondrial proteins (PGC-1α, NRF1, TFAM, ERRα), mitochondrial membrane potential (ΔΨm), mitochondrial ATP levels and mitochondrial mass were higher in drug-resistant MM cells and lower in drug-sensitive MM cells. After silencing CRBN, expression of mitochondrial proteins, ΔΨm, mitochondrial ATP level and mitochondrial mass were significantly increased in drug sensitive MM cells. Conversely, adenovirus-infected CRBN lead to decreased mitochondrial function.
Conclusion: These findings suggest that CRBN is a novel therapeutic target for overcoming drug resistance in MM, via the mediation of mitochondrial dysfunction. Furthermore, combined CRBN and mitochondria marker evaluation are more effective in patients suited for thalidomide therapy.

Keywords: CRBN, multiple myeloma, thalidomide, mitochondria

Introduction:
Multiple myeloma (MM) is an incurable plasma cell malignancy characterised by the proliferation and accumulation of tumor cells in the bone marrow. In the Western countries, MM accounts for approximately one percent of all cancerrelated deaths, and around 15-20% of all hematological malignancies.

Over half a decade ago, thalidomide was primarily used to treat insomnia and to alleviate morning sickness in pregnant women, but its teratogenic effects resulted in a wide variety of birth defects and eventually, thalidomide was banned for use in pregnant women. However, in the mid-1990's thalidomide was found to be effective in the treatment of MM and reported to have a multitude of activities, including immunomodulatory, antiangiogenic and cytotoxicity. Subsequently, Lenalidomide was approved for use in combination with dexamethasone in patients with MM who had received at least one prior treatment.

Cereblon (CRBN) is a thalidomide binding protein and appears to play an important role in mediating antimyeloma effects, associated with the teratogenicity and cytotoxicity of ImiDs. Furthermore, CRBN plays a major role in cancer cell biology. In 2010, Ito et al. identified CRBN is a primary target that mediates the teratogenicity of thalidomide, linked to cytotoxicity and highly efficient anti-myeloma activity. Thalidomide has been demonstrated to bind to CRBN at specific sites, which leads to the inhibition of E3 ubiquitin ligase (Cul4) complex activity. CRBN can also be a marker for high and low-risk patients, as well as drug sensitive and insensitive patients. For example, lower levels of CRBN have been observed in MM patients who are lenalidomide-resistant or MM patients with stable or progressive disease, whereas higher levels of CRBN are observed in MM patients who respond treatment and MM patients with complete remission. Furthermore, CRBN depletion is initially cytotoxic to the myeloma cells, surviving cells with stable CRBN depletion become highly resistant to IMiD agents.

Mitochondria are intracellular dynamic organelles that regulate energy metabolism, cell death, and signaling pathways that are important for cell proliferation and differentiation. Dysfunction of mitochondria is considered a

biomarker of metabolic diseases. However, CRBN and mitochondria biomarkers in MM cells have not been reported. Thus, we investigated the correlation between CRBN and mitochondrial function in MM cells. In this study, we hypothesized that CRBN can regulate mitochondria biogenesis and function, therefore thalidomide sensitivity may be related to the altered of mitochondrial activity. To understand the role of mitochondria in IMiDs-treated MM cell lines (such as drug-resistant KMS20 and drug-sensitive KMS26), we evaluated several parameters related to mitochondrial biogenesis and mitochondrial activity. We found evidence that CRBN negatively regulates mitochondrial function and hence is an effective target to overcome drug resistance in MM.

Result:

A. Low CRBN associated with thalidomide resistant MM cells: To investigate the correlation between CRBN protein expression and thalidomide resistance in MM cells. We used three MM cell lines-KMS20, KMS26 and KMS28BM examined their resistance capacity to thalidomide using cell viability assay. The previous study showed that KMS20 cells were resistant, KMS26 and KMS28 cells were sensitive to bortezomib-mediated cell death. We checked the link of cell death and CRBN expression in MM cells in the response to IMiDs drugs, especially thalidomide and lenalidomide. KMS cells were cultured at various concentrations of thalidomide, times of thalidomide and analyzed CCK-8 cell viability assay. As shown in figure 1A cell death was significantly reduced in KMS26 and KMS28BM cells. Therefore, KMS cells were treated with 5uM thalidomide during different hours KMS26 and KMS28BM cells also significantly decreased cell survival compared with that of KMS20 cells (Fig.1B). Next, we checked CRBN protein expression in KMS20, KMS26 and KMS28BM cells by western blotting. As shown in figure 1C KMS20 cells have significantly lower CRBN compared with KMS26 and KMS28BM cells. These data are confirmed by the previous study, very low CRBN expression is correlated with drug resistance in MM cell lines. Figure 1D indicate that KMS20 cells, which express lower levels of CRBN, were resistant to thalidomide, whereas KMS26 and KMS28BM cells, which express higher levels of CRBN, were sensitive to thalidomide. In contrast, KMS20 cells exhibited resistance to cell death by thalidomide. Even, a high concentration of thalidomide in CRBN-low KMS20 cells cannot be efficiently killed (Fig.2A). In fact, lenalidomide appears to more effective at a concentration much lower than that of thalidomide. As shown figure 2B, lenalidomide-treated KMS cells are more effectivily reduced cell viability. These results clearly indicate that whereas KMS26 and KMS28 are a thalidomide-sensitive MM cell line, KMS20 cells have acquired resistance to thalidomide. Moreover, these results imply that very low CRBN expression in KMS cell line is associated with IMiDs drug resistance.

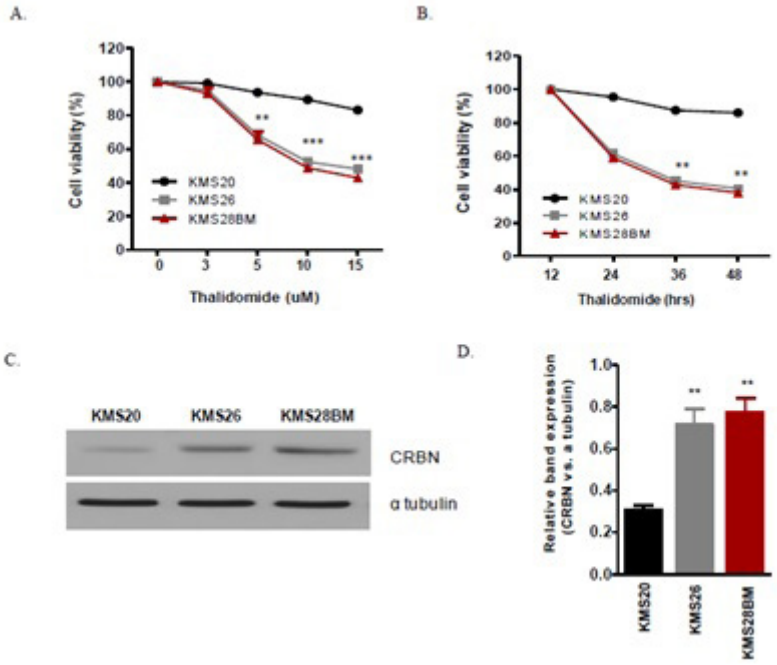


Figure 1. IMiDs induced cell death and CRBN protein expression in MM cells

(A) KMS cells were indicated at different doses and for (B) different period of time with thalidomide and cell viability was determined by CCK-8 assay. (C) CRBN protein expression in KMS cells, (D) comparison of CRBN expression in KMS cells, the intensity of the α tubulin was considered as 1.0 and relative intensity of CRBN band. *p<0.05, **p<0.01, ***p<0.001

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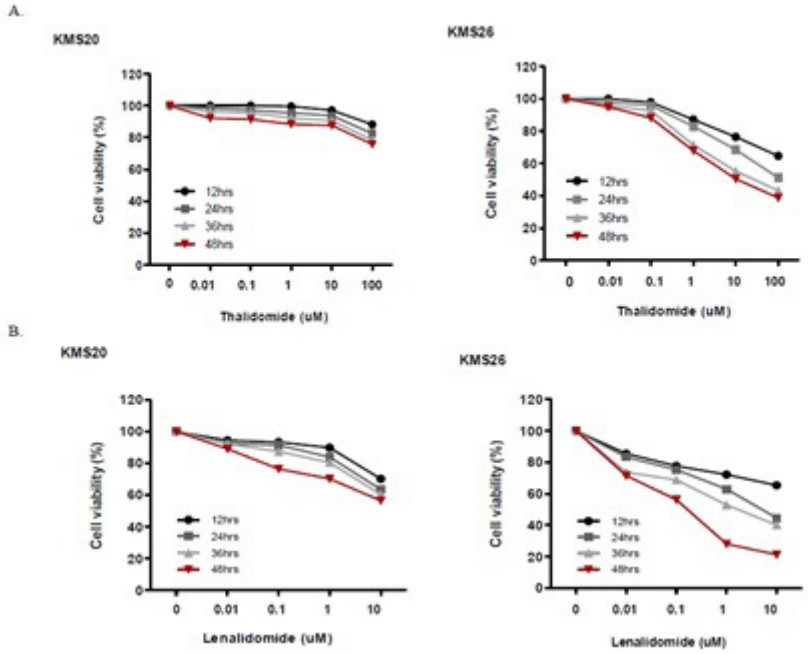


Figure 2. KMS 20 is resistant to IMiDs

(A) KMS cells were indicated at different doses and different period of time with thalidomide (B) KMS cells were indicated at different doses and different period of time with lenalidomide, cell viability was determined by CCK-8 assay

B. Mitochondrial proteins and genes highly expressed in drug-resistant MM cells

To analyzed the basal expression of mitochondrial genes associated with mitochondrial function and mitochondrial biogenesis in KMS cells. Several genes were differentially expressed in both cell lines (Fig.3A). First, we checked CRBN and some specific mitochondrial genes (PGC1a, TFAM, CYPD, SOD2 and MCU) associated with ΔΨm, ROS and energy metabolism in KMS20 cells and KMS26 cells. The expression of CYPD, SOD2 and MCU, which are related to maintenance of the ΔΨm, elimination of ROS and Ca2+ influx into mitochondria. Figure 3A showed drug sensitive cells expressed significantly higher CRBN and low mitochondrial genes. However, in drug resistant KMS20 cells highly expressed PGC1a and TFAM genes compared with KMS26 and KMS28BM cells. Moreover, in western blot analysis, the pattern of protein expression of these genes demonstrated a similar result (Fig.3B). Previous investigations showed that CRBN level is increased after thalidomide and lenalidomide treatment in the MM cell lines, which increases the drug sensitivity of multiple myeloma cell lines. Likewise, our data showed CRBN level increased after thalidomide treatment and decreased AMPKa1 and mitochondrial proteins (Fig.3C). These results matched with the previous study that CRBN as a negative regulator of AMPK signaling. Furthermore, mitochondrial proteins expressed lower in thalidomide sensitive KMS26 cells compared with thalidomide resistant KMS20 cells. Next, we checked with various concentrations of thalidomide treatment in KMS cells. CRBN levels dramatically increased with 10uM thalidomide treatment in both cells. However, AMPKα1, PGC1α, TFAM, ERRα and NRF1 proteins slightly decreased in KMS cells, notably in drug- sensitive cells (Fig.3C). From these results, I assume that chemo-resistance of MM patients is regulated according to the specific expression of genes associated with mitochondrial function and CRBN negatively regulates mitochondria biogenesis.

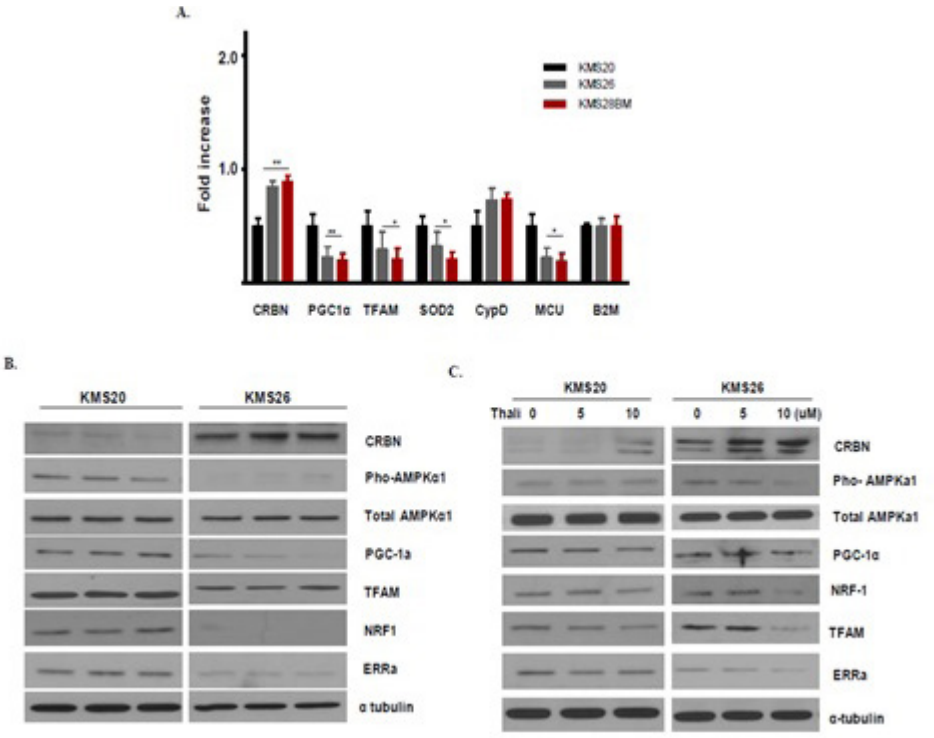


Figure 3. Mitochondrial biogenesis related genes and protein expressions in MM cells.
(A) Gene expressions measured by qPCR, (B) protein expression by western blotting using indicated antibodies, (C) KMS cells were treated with thalidomide at the indicated doses for 36h and were subjected to western blotting using indicated antibodies.

C. Mitochondrial membrane potential and ATP levels higher in drug-resistant MM cells

To investigate the connection between mitochondrial activity and thalidomide
resistance in MM, we examined mitochondrial functions, such as ΔΨm, mitochondrial ROS, ATP production and mitochondrial mass. First, we checked the basal level of ΔΨm in the three cell lines. The ΔΨm was measured using TMRE, which accumulates in the mitochondria via a ΔΨm driven process. Because uptake of TMRE into mitochondria depends on ΔΨm, a collapse of ΔΨm is associated with a decrease in the fluorescence intensity of TMRE. Thalidomide-treated KMS cells exhibited a depolarization of ΔΨm and that this response is differently induced by thalidomide. The ΔΨm was greater in thalidomide-resistant KMS20 cells than in thalidomide-sensitive KMS26 and KMS28BM cells (Fig.4A) and TMRE intensity higher in KMS20 cells (Fig.4B). However, thalidomide treated KMS26 cells TMRE intensity decreased more than 70% and KMS20 cells exhibited a depolarization of ΔΨm and that this response increased according to the dosage of thalidomide (Fig.4C). Next, we measured the mitochondrial ROS level in KMS cells, using an oxidant-sensitive fluorescent dye MitoSox Red by flow cytometric and confocal microscopy. Figure 5A showed that drug sensitive KMS26 and KMS28 cells have ROS levels higher than drug resistant KMS20 cells, likewise confocal microscopy (Fig.5B) analyses of red fluorescence highly indicated than resistant cells. The KMS26 cells resulted in a significantly increase of mitochondrial ROS level by thalidomide treatment (Fig.5C). To better characterize the mitochondrial energy metabolism underlying the susceptibility of KMS cells to thalidomide, we analyzed their ATP concentrations. We measured cellular and mitochondrial ATP (mtATP) level by Mitochondrial ToxGlo™ assay. KMS20 cells cellular and mtATP (Fig.6A) levels were significantly greater in KMS20 cells than drug-sensitive KMS26 and KMS28BM cells. Furthermore, the mtATP concentrations in KMS26 and KMS28BM cells were decreased by thalidomide treatment, but not much affected in KMS20 cells (Fig.6B). Finally, we measured mitochondrial mass using NAO staining in KMS cells. Drug sensitive KMS26 cells NAO fluorescence slightly decreased by thalidomide treatment, but no changed in KMS20 cells. These all data matched with our previous study that mitochondrial modulation decreases the bortezomib-resistance in MM cells. Thus, our results suggest that mitochondrial functional differences including ΔΨm, ROS production, ATP production and mitochondrial mass were involved in the susceptibility of MM cells.

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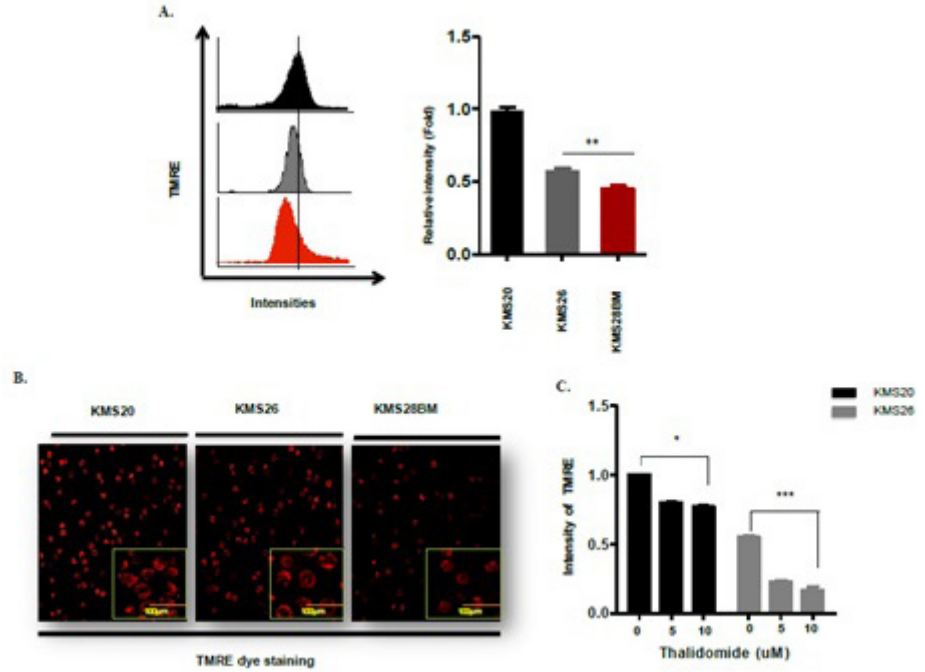


Figure 4. Mitochondrial membrane potential higher in drug-resistant MM cells

(A) mitochondrial membrane potential by FACS Calibr (B) TMRE statining of ΨΔm by confocal microscopy, (C) thalidomide treated KMS cells ΨΔm by FACS Calibr *p<0.05, **p<0.01, ***p<0.001

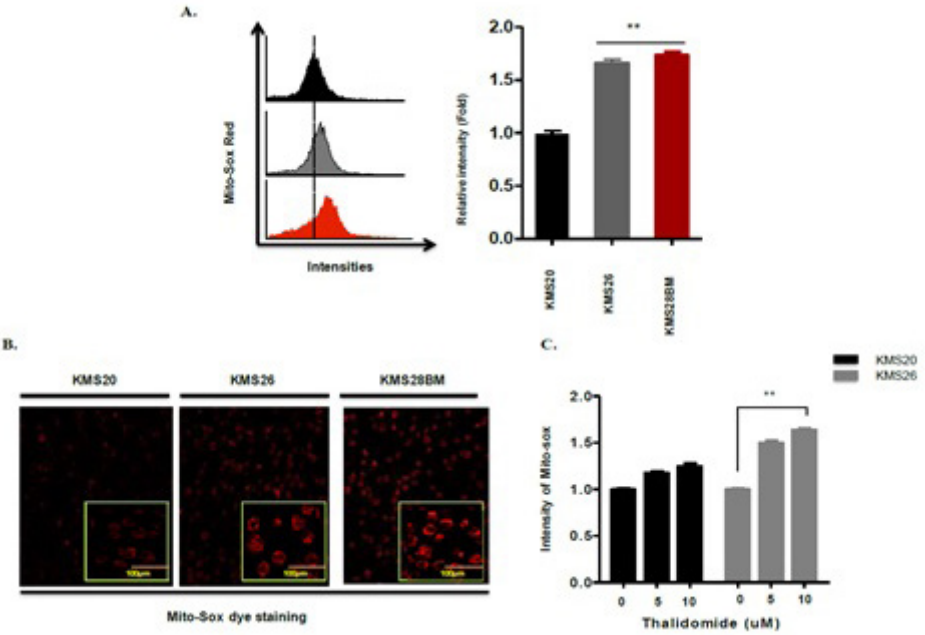


Figure 5. Mitochondrial ROS level lower in drug-resistant MM cells

(A) mitochondrial ROS level in KMS cells using a FACS Calibr and (B) Confocal microscopy. (C)-thalidomide treated KMS cells ROS level measured by FACS calibr *p<0.05, **p<0.01, ***p<0.001

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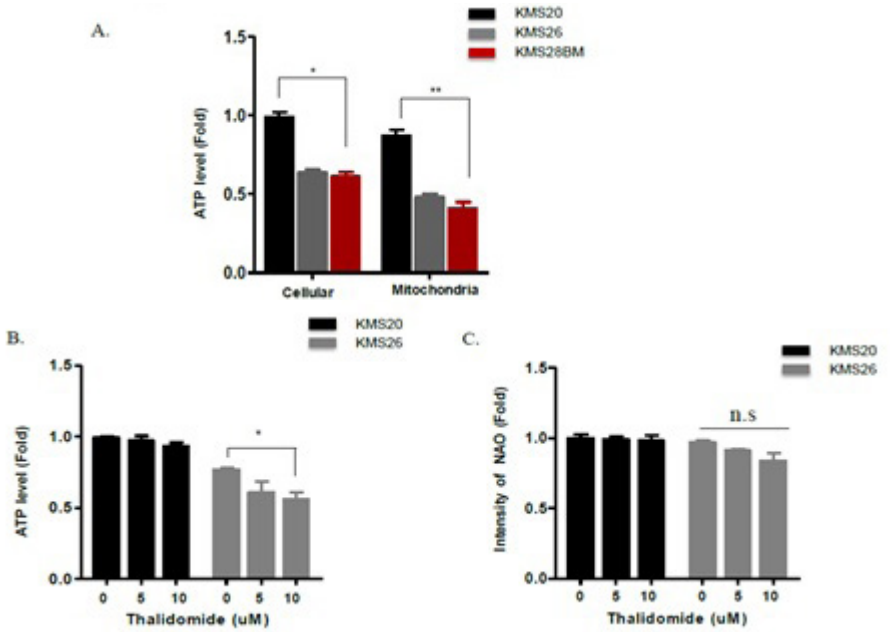


Figure 6. Mitochondrial ATP production higher in thalidomide-resistant MM cells

(A) Cellular and mitochondrial ATP level in KMS cells (B) thalidomide treated KMS cells ATP level by Mitochondrial ToxGlo assay, (C) thalidomide treated KMS cells mitochondrial mass by NAO staining by a FACS Calibr *p<0.05, **p<0.01, ***p<0.001

D. Mitochondrial protein expression increased in knockdown MM cells

Our previous data (Fig.1) showed that KMS26 cells expressed higher CRBN than KMS20 cells. So, we silenced CRBN by shRNA (gene expression knockdown, CRBN shRNA) in drug sensitive KMS26 cells. First, we measured knockdown KMS26 cells, cell proliferation by CCK-8 assay. In fact, CRBN knockdown reduces lenalidomide sensitivity of a multiple myeloma cell line. Even, our results also showed CRBN shRNA have a significant reduction of cell viability which is shCRBN can inhibit cell proliferation (Fig.7A) and changed to the drug resistant condition. Then, we checked CRBN protein expression by western blotting (Fig.7B and Fig.7C). KMS26 cells were transfected with the CRBN shRNA following the CRBN protein expression significantly reduced. Furthermore, immunofluorescence results presented in figure 8A showed that intensity of CRBN was dramatically decreased in the shCRBN transfected KMS26 cells, as compared with the normal KMS26 cells. Next, we checked mitochondrial specific proteins under shCRBN transfection. We clearly observed that mitochondrial proteins expressions were significantly higher than during normal condition (Fig.8B). Our previous data showed (Fig.3C) 10uM thalidomide treated cells CRBN level dramatically increased and mitochondrial protein reduced. However, shCRBN transfected KMS26 cells treated with 10uM thalidomide slightly increased CRBN level and slowly decreased mitochondrial proteins, such as PGC1α, TFAM and ERRα with same dosage thalidomide treatment (Fig.8D).

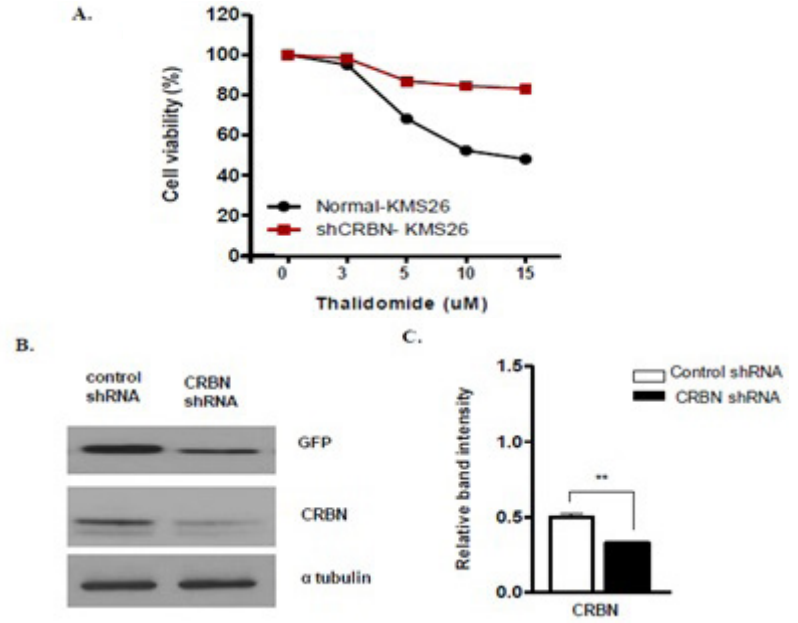


Figure 7. CRBN knockdown induces cell death in KMS26 cells

(A) cell viability after shRNA transfection determined by CCK-8 assay (B) CRBN protein expression by western blotting, (C) the intensity of the α tubulin was considered as 1.0 and relative intensity of CRBN band *p<0.05, **p<0.01, ***p<0.001

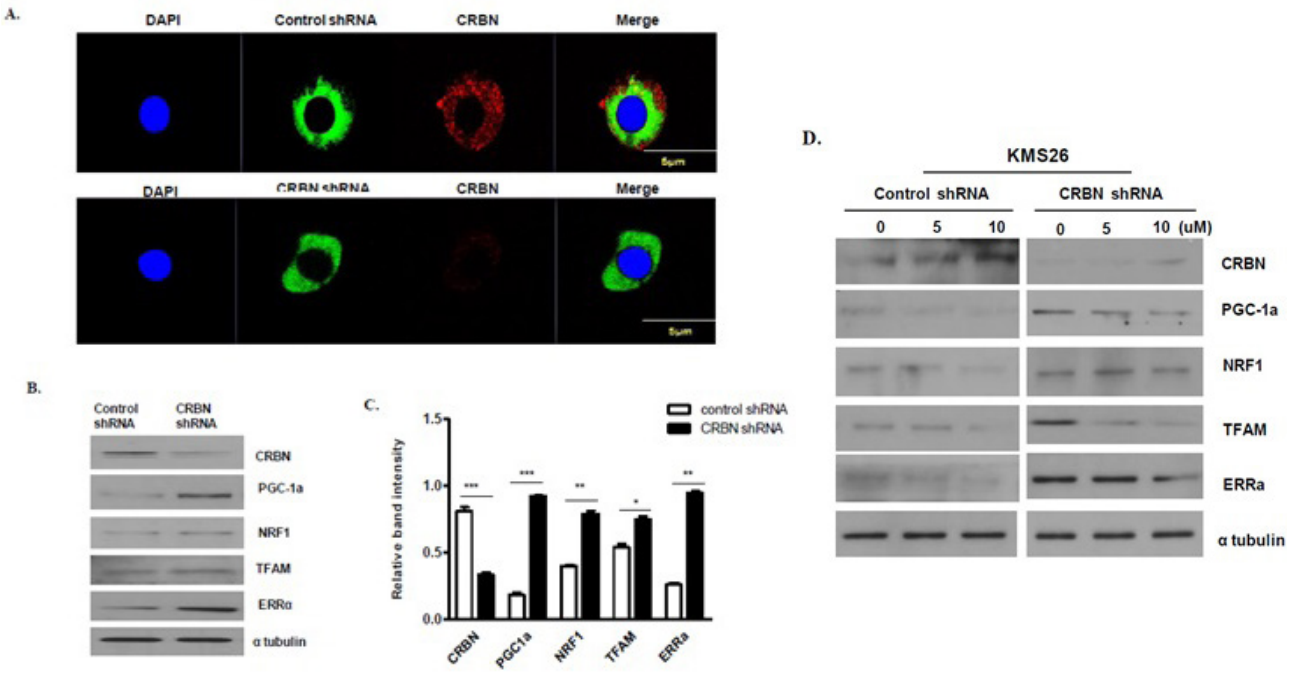


Figure 8. CRBN knockdown induces protein expression in KMS26 cells

(A) Immunocytochemistry analysis after transfection with DAPI staining (B) CRBN and mitochondrial protein expression, (C) Knock down KMS26 cells were treated with thalidomide at the indicated doses for 36h and were subjected to western blotting using indicated antibodies, (d) mitochondrial protein expression analyzed by western blotting. *p<0.05, **p<0.01, ***p<0.001

E. CRBN downregulation leads to increase mitochondrial function

To determine the regulators of mitochondrial activity, we analyzed the effect of shCRBN on basal level of ΔΨm, mitochondrial ROS, mitochondrial ATP and mitochondrial mass by flow cytometry. The levels of ΔΨm were measured using

TMRE staining, as shown figure 9A increased ΔΨm in knockdown KMS26 cells compared to normal condition. However, significantly reduced ΔΨm intensity with various concentration of thalidomide (Fig.9B). Our previous data showed (Fig.5A) mitochondrial ROS dramatically increased in KMS26 cells. But, after transfected knockdown KMS26 cells, ROS slightly increased with 10uM thalidomide treatment (Fig.9C). Also, a mitochondrial specific-Mitosox dye of red fluorescence highly indicated during increasing concentration of thalidomide (Fig.9D). In addition, mitochondrial mass with NAO staining in shCRBN transfected KMS26 cells mitochondrial mass increased with 5uM and 10uM thalidomide (Fig.10A). Finally, we checked mitochondrial ATP level in knockdown KMS26 cells. As shown in figure 10B, mitochondrial ATP increased with different concentrations of thalidomide. Taken together, these data suggested that CRBN downregulation leads to increase mitochondrial function in KMS26 cells.

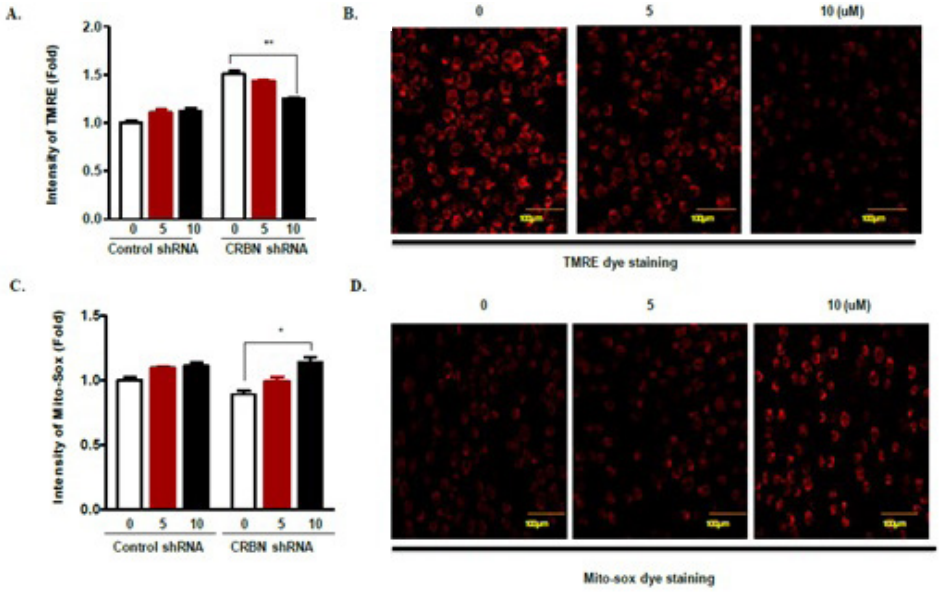


Figure 9. CRBN knockdown induces mitochondrial function in thalidomide sensitive KMS26 cells

(A) mitochondrial membrane potential in normal and shCRBN transfected KMS26 cells treated with thalidomide analyzed by FACS calibr, (B) analyzed by confocal microscopy, (C) normal and shCRBN transfected KMS26 cells treated with thalidomide at the indicated doses subjected to flow cytometry after staining with MitoSox red dye (D) analyzed by confocal microscopy *p<0.05, **p<0.01, ***p<0.001

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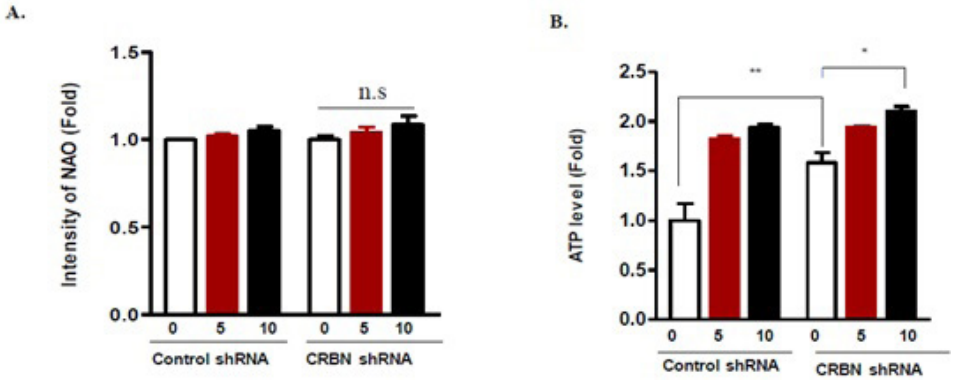


Figure 10. Inceased mitochondrial mass and ATP level in shCRBN transfected KMS26

(A) mitochondrial mass in normal shCRBN transfected KMS26 cells treated with thalidomide analyzed by FACS calibr (B) mitochondrial ATP in normal and shCRBN transfected KMS26 cells treated with thalidomide by Mitochondrial ToxGlo assay. *p<0.05, **p<0.01, ***p<0.001

F. Mitochondrial protein expression decreased in adenovirus-infected MM cells

Previously, we clearly investigated KMS20 cell has low CRBN compared with KMS26 and KMS28BM cells. In this case, we used adenovirus for the gain of function of CRBN expression. First, we infected by adenovirus into drug-resistant KMS20 cells and checked again CRBN expression and mitochondrial protein expression. Very interestingly, drug resistant KMS20 cells infected with adenovirus for 72 hr, KMS20 cells viability significantly reduced (Fig.11A). It's matched with previous studies reported that adenovirus-mediated overexpression in cancer cells leads to increase the number of apoptotic cells. Then, we checking CRBN level by western blotting used CRBN antibody. As shown figure 11B CRBN level significantly increased after adenovirus infection and figure 11C showed relative band intensity. Next, we checked mitochondrial protein expressions by western blotting. As previously known, CRBN overexpression reduces the expression of PGC1α and SRE-binding protein. Similarly, when CRBN level increased, PGC1a, NRF1, TFAM, ERRa proteins expression conversely reduced after adenovirus infection (Fig.12A).

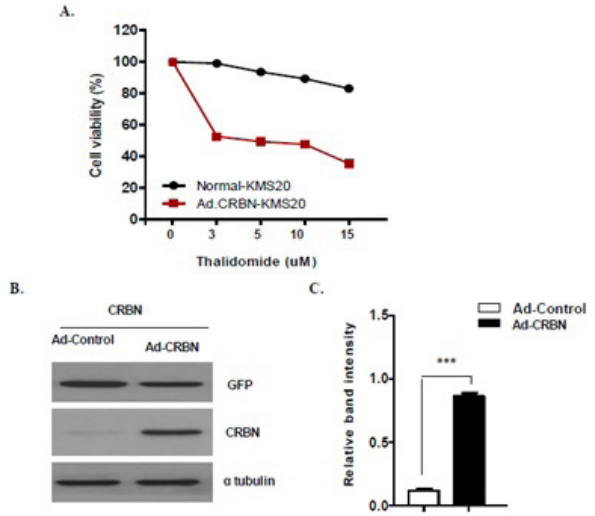


Figure 11. CRBN overexpression blocking cell death

(A) cell viability after adenovirus infection checked by CCK-8 assay, (B) CRBN expression level by western blotting, (C) relative band intensity of CRBN expression by Image J analysis. *p<0.05, **p<0.01, ***p<0.001

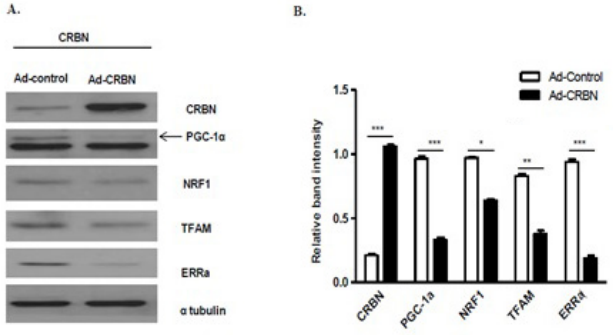


Figure 12. CRBN overexpression reduced protein expression in thalidomide resistant KMS20 cells

(A) CRBN and mitochondrial protein expression by western blotting, (B) relative band intensity of CRBN and mitochondrial proteins expression by Image J analysis. *p<0.05, **p<0.01, ***p<0.001

G. Adenovirus-infected CRBN leads to decrease mitochondrial function

To check the regulators of mitochondrial activity, we analyzed the AdCRBN infected KMS20 cells for ΔΨm, mitochondrial ROS, mitochondrial ATP and mitochondrial mass by flow cytometry. Figure 13A showed AdCRBN infected KMS20 cells Ψm slightly decreased with thalidomide drug treatment, and analyzed by confocal microscopy showed TMRE dye fluorescence (Fig.13B) reduced. However, mitochondrial ROS level (Fig.13C) significantly increased compared with control group. Therefore, MitoSox red dye fluorescence also increased during confocal imaging (Fig.13D). Next, we checked mitochondrial ATP level (Fig.14A) and mitochondrial mass (Fig.14B) by FACS analysis. But, ATP and the mitochondrial mass slightly decreased with thalidomide treatment. Taken together, these data suggested that CRBN overexpression reduced mitochondrial function in thalidomide resistant KMS20 cells.

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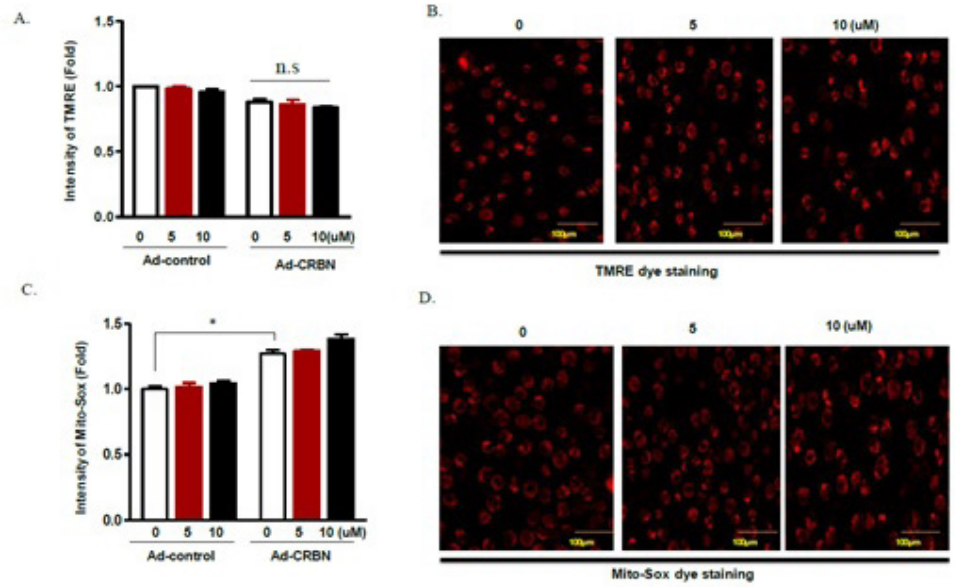


Figure 13. CRBN overexpression reduced mitochondrial function in thalidomide-resistant KMS20 cells

(A) mitochondrial membrane potential in normal and ad-CRBN infected KMS20 cells treated with thalidomide analyzed by FACS calibr, (B) analyzed by confocal microscopy, (C) normal and ad-CRBN infected KMS20 cells were treated with thalidomide at the indicated doses subjected to flow cytometry after staining with MitoSox red dye (D) analyzed by confocal microscopy. *p<0.05, **p<0.01, ***p<0.001

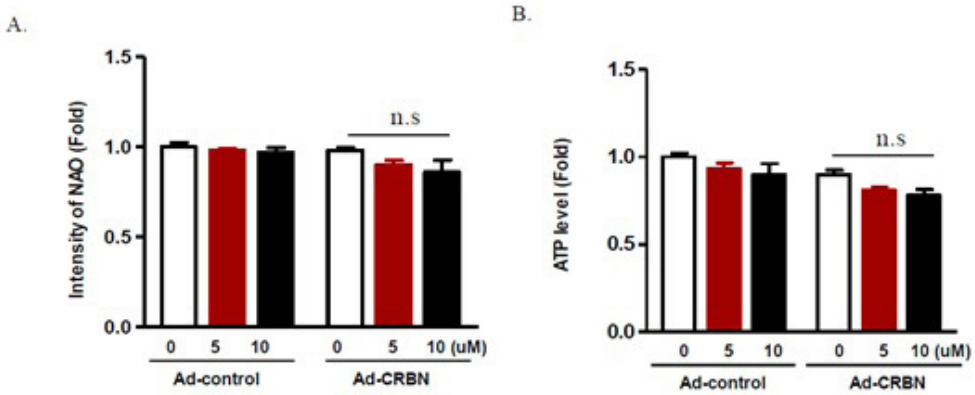


Figure 14. Decreased mitochondrial mass and ATP level in AdCRBN infected KMS20 cells

(A) Mitochondrial mass in normal and ad-CRBN infected KMS20 cells treated with thalidomide analyzed by FACS calibr (B) mitochondrial ATP in normal and ad-CRBN infected KMS20 cells treated with thalidomide by Mitochondrial ToxGlo assay

Conclusion: In this study, we suggest that CRBN is a novel therapeutic target for overcoming drug resistance in MM patients, via the mediation of mitochondrial dysfunction. Furthermore, combined CRBN and mitochondrial marker evaluation are more effective in patients suited for thalidomide target therapy. In future, further studies are necessary to improve our understanding of the molecular mechanisms underpinning the role of CRBN in the drug resistance of MM cells.

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Journal of Clinical Medicine - 5.688
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BMB Reports - 3.08

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