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14. ABSTRACT: Dysregulation of apoptosis is an important mechanism underlying the resistance to radio- or chemotherapy of androgen-insensitive prostate cancer. BI-1 and BAR were identified as inhibitors of a pro-apoptotic Bcl-2 family member, Bax, identified by a functional yeast screen. Both BI-1 and BAR are membrane proteins located at endoplasmic reticulum (ER). The importance of BI-1 in prostate cancer is underscored by its specific up-regulation in malignant cells. Furthermore, reduction of BI-1 expression by small interference RNA (siRNA) causes significant increase of spontaneous cell death in prostate carcinoma cells. This research project is about a regulatory protein network involving BI-1, BAR, Bap31, and Bcl-2. Using a coimmunoprecipitation assays, we demonstrated that BAR is bound to BI-1, Bap31, and Bcl-2, forming a multi-protein complex at endoplasmic reticulum. BAR is a multifunctional protein with a coiled-coiled domain, a SAM domain, and a predicted E3 ubiquitin ligase RING domain. The RING domain is proved to be essential for the E3 ubiquitin ligase activity of BAR. Acting as an E3 ubiquitin ligase, BAR specifically reduces the protein level of BI-1, but not Bap31 or Bcl-2. On the other hand, Bap31 was found to specifically increase the stability of BAR by decreasing proteosome-dependent degradation of BAR. A regulatory hierarchy seems to exist that Bap31 regulates the protein stability of BAR, an E3 ubiquitin ligase, which in turn modulates the stability of BI-1 in the protein complex.					
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Introduction

Dysregulation of apoptosis is an important mechanism underlying the resistance to radio- or chemotherapy of androgen-insensitive prostate cancer. Regulation of apoptosis relies on the intricate interplay of anti-apoptotic and pro-apoptotic protein networks in the cells, in which Bcl-2 family members play an essential role. Using a functional screen in yeast, BI-1 (Bax inhibitor –1) and BAR (Bifunctional Apoptosis Regulator, also BI-2) were identified as inhibitors of a pro-apoptotic Bcl-2 family member, Bax [1-5]. Both BI-1 and BAR are membrane proteins located at endoplasmic reticulum (ER). The importance of BI-1 in prostate cancer is underscored by its specific up-regulation in malignant cells. Two independent studies to search for genes differentially expressed in normal and malignant prostate tissues have identified BI-1 as highly and specifically expressed in malignant samples [6, 7]. Quantitative RT-PCR and *in situ* hybridization experiments confirmed that up-regulation of BI-1 is restricted in malignant epithelial cells. Furthermore, reduction of BI-1 expression by small interference RNA (siRNA) causes significant increase of spontaneous cell death in prostate carcinoma cells [6]. In addition, BI-1 was found to be up-regulated in a number of tumors including breast cancer [8], glioma [9], and anaplastic large cell lymphoma [10].

This research project is about a regulatory protein network involving BI-1, BAR, Bap31, and Bcl-2. Using coimmunoprecipitation assays, we demonstrated that BARdR (without RING domain) is bound to BI-1, Bap31, and Bcl-2 when coexpressed in 293T cells. Reciprocally, Bap31 was also found to coimmunoprecipitate with BI-1, BARdR, and Bcl-2 in 293T cells, suggesting a protein complex formed by BI-1, BAR, Bap31, and Bcl-2 at endoplasmic reticulum. BAR is a multi-domain protein with a pseudo-DED domain (with remote homology to Death Effector Domain), a SAM domain, and a predicted E3 ubiquitin ligase RING domain. The RING domain is proved to be essential for the E3 ubiquitin ligase activity of BAR. BAR full length formed multiple ubiquitin-conjugated forms and was greatly stabilized when treated with proteasome inhibitor MG132 *in vivo*. Deletion of RING domain in BAR (BARdR) greatly increases protein stability and makes the protein insensitive to MG132 treatment. Consistently, only BAR full length, not BARdR, can act as an E3 ubiquitin ligase for polyubiquitination

of itself in an *in vitro* ubiquitination assay. Acting as an E3 ubiquitin ligase, BAR specifically reduces the protein level of BI-1, but not Bap31 or Bcl-2, when coexpressed in 293T cells. On the other hand, Bap31 was found to specifically increase the stability of BAR by decreasing proteasome-dependent degradation of BAR. Therefore, a regulatory hierarchy seems to exist that Bap31 regulates the protein stability of BAR, an E3 ubiquitin ligase, which in turn modulates the stability of BI-1 in the protein complex. Further investigation of targets of BAR E3 ubiquitin ligase activity will provide insights into the regulatory roles of this protein complex in apoptotic pathways and afford new strategies for treating prostate cancer.

Body

Measurement of Ca²⁺ homeostasis in BI-1 knock-out cells reconstituted with BI-1

BI-1 is a structurally and functionally conserved protein that was reported to be specifically involved in ER stress related cell death pathways. It was previously reported in our laboratory that BI-1 knockout MEF cells have higher ER calcium content than wild type MEF cells [2]. This led us to hypothesize that BI-1 regulate ER stress related cell death through regulation of ER calcium homeostasis, since altered Ca²⁺ signaling is one important factor contributing to ER stress pathways. Previous studies compared calcium release induced by thapsigargin (TG), an ER calcium pump inhibitor, in BI-1^{-/-} and BI-1^{+/+} MEF cells. To further test the hypothesis, it is important to reconstitute BI-1 into BI-1^{-/-} MEF cells to see if BI-1 can restore the Ca²⁺ homeostasis, since ER Ca²⁺ content and release is a dynamic parameter that depends on various factors including cell types, physiological status, culture history, and growth state.

Ca²⁺ releases in single cells were measured using Fura 2 fluorescent dye, which is a sensitive method for measuring calcium dynamics. One difficulty encountered during my studies is that the measurement variation between different cell culture dishes could be quite significant at some times (See Figure 1), complicating the explanation of measurement results. In Figure 1, the same cells were cultured, transfected, and treated in exactly the same way, however, the peak calcium release had almost three-fold difference between two culture dishes. To overcome this difficulty, I established an experimental paradigm that measures cellular Ca²⁺ responses within the same culture dish. The gene(s) of interest was cotransfected with a red fluorescent gene, DsRed, to label transfected cells. Ca²⁺ responses in transfected cells (red) and non-transfected cells (non-red) were then measured in the same culture dish, comparing neighboring cells with the same culture condition and microenvironment. I tested and confirmed that both empty vector and DsRed had no effect on either Fura 2 fluorescence itself or TG-induced Ca²⁺

release. Using this new strategy, Ca^{2+} releases induced by TG in BI-1 transfected cells (red) and non-transfected cells (non-red) were compared in Figure 2 (data from two representative dishes were shown). No difference in TG-induced Ca^{2+} release in terms of release rate, release peak, and clearance rate was observed in BI-1 transfected cells vs. non-transfected cells. With repeated trials, I was not able to confirm the previous observation using this new and more reliable experimental paradigm. I therefore looked for other avenues to investigate the mechanism of BI-1 to regulate cellular functions. I looked for proteins associated with BI-1 and studied how their interactions are involved in cellular regulation.

Multi-protein complex including BI-1, BAR (BI-2), Bap31, and Bcl-2 is formed at endoplasmic reticulum

It is an interesting observation that the two Bax inhibitors identified by the yeast lethal screen, both BI-1 and BAR, are located at endoplasmic reticulum. Furthermore, they are both associated with Bcl-2 but not Bax, suggesting they regulate the function of Bax through an indirect pathway. It is possible that both of them are involved in an ancient ER-related cell death pathway, which is well conserved through yeast to mammals. BAR associates with and shares homology in the region of the pseudo-DED domain with another ER membrane protein called Bap31 [4], which is involved in cell death regulation and cross-communication between endoplasmic reticulum and mitochondria [11]. Since the communication between endoplasmic reticulum and mitochondria is essential for the function of BI-1 and BAR, it is possible that BI-1, BAR, Bap31, and Bcl-2 form a multi-protein complex to coordinate this important function. To test if the multi-protein complex forms in cells, different combinations of tagged proteins were cotransfected into 293T cells, coimmunoprecipitated with appropriate antibodies, separated by SDS-PAGE, and detected by western blot (Figure 3 A). It was shown that BI-1 was coimmunoprecipitated by both BAR and Bap31, and BAR is coimmunoprecipitated with Bap31 and Bcl-2 as well, supporting the hypothesis that these four proteins form a multi-protein complex at the endoplasmic reticulum.

To determine the domain of BAR involving in the interaction between BAR and Bap31, the interaction between Bap31 and several BAR mutants were studied (Figure 3 B). BARdR (deletion of RING domain), BARdRdTM (deletion of RING and transmembrane domain), BARdRdDEDdTM (deletion of RING, transmembrane, and DED domain) were cotransfected with Flag-tagged Bap31, coimmunoprecipitated with anti-Flag antibody, and detected by anti-BAR antibody. It was shown that RING domain was not required for the interaction of BAR and Bap31 as expected. Removal of transmembrane domain alone did not affect the binding of these two proteins, but deletion of both transmembrane and DED domain led to the loss of the association. Since I had difficulty expressing a BAR mutant with only DED and RING domain deleted, it remains to see whether deletion of DED domain alone can abolish the interaction between BAR and Bap31.

Bap31 has multiple protein interacting domains

Should a multi-protein complex need to be formed, a scaffold protein with multiple interacting domains is required. Our studies revealed that Bap31 could interact with proteins through multiple domains, namely the N-terminal transmembrane domain (Bap31-p20) and the DED domain resided at the cytoplasmic tail of the protein. The crystal structure of the cytoplasmic tail of Bap31 (aa¹²³ – aa²⁴⁶, named DED_{Bap31}) was partially solved by a collaborator at Burnham Institute. It was found that purified DED_{Bap31} automatically forms a dimer, which is stabilized by the hydrophobic interactions of four critical leucine residues in the pseudo-DED domain. To determine if the interaction between DED domains of Bap31 occurs *in vivo*, a Bap31 mutant (Bap31mt) was made by simultaneously changing the four critical leucine residues to alanines and specifically weakening the interaction between DED domains. Coimmunoprecipitation studies (Figure 4) showed that wild type Bap31 could bind to Bap31, Bap31-p20, and DED_{Bap31}, suggesting that both transmembrane and DED domain contribute to the interaction. The loss of binding is specifically limited to Bap31mt and DED_{Bap31}, and Bap31mt still maintains

binding to Bap31 and Bap31-p20. This further confirmed that Bap31 interacts with other proteins through two independent domains, the transmembrane and DED domain. To the similar accounts, BAR may also interact with other proteins through its transmembrane domain and DED domain. Bap31 was reported to be cleaved by activated caspase 8 when treated with death receptor antibody (CH11) and cycloheximide (CHX), and the cleavage product promotes cell death [11,12]. The mutation in DED domain of Bap31 makes it more sensitive to CH11/CHX induced cleavage, suggesting that the DED domain may be involved in regulating the interaction between Bap31 and caspase 8 (Figure 5). The CH11/CHX induced cleavage of Bap31 is caspase-dependent because caspase inhibitor, zVAD, can inhibit the cleavage.

RING domain of BAR is essential for the E3 ubiquitin ligase activity

A RING E3 ubiquitin ligase domain at the N-terminal of BAR is predicted from an NCBI domain search algorithm. An E3 ubiquitin ligase is an enzyme that facilitates the last step of ubiquitination process and converts specific target proteins into ubiquitinated forms. Some E3 ubiquitin ligases target themselves to promote self-ubiquitination and self-degradation. The first observation that BAR mutation without RING domain (BARdR) is much more stable than the full-length protein (Figure 6 A) suggests that the RING domain of BAR is an authentic E3 ubiquitin ligase domain. Further evidence came from cells transfected with BAR or BARdR that were treated with proteasome inhibitor, MG132. Only BAR, but not BARdR, was stabilized by MG132 treatment and formed multiple ubiquitinated forms at the higher concentration of MG132 treatment (Figure 6 A). To directly test the E3 ligase activity of BAR, the protein was immunoprecipitated and incubated with E1, E2, ubiquitin (Ub), and ATP in an *in vitro* ubiquitination assay (Figure 6 B). Only BAR, but not BARdR, showed weak but detectable ubiquitinated product in the *in vitro* assay, suggesting that the RING domain is the essential domain for E3 ligase activity. To further test the *in vivo* E3 ligase activity of BAR, ubiquitinated proteins that are associated with BAR or BARdR were pulled down by immunoprecipitated and detected by western blot. If BAR functions as an E3 ligase to

facilitate the ubiquitination of target proteins that associate with it, we are expected to see more ubiquitinated proteins in BAR associating proteins than BARdR associating proteins. Figure 7 showed more ubiquitinated proteins associated with BAR than those of BARdR with MG132 treatment, further providing evidence that BAR functions as an E3 ligase *in vivo*. Putting together, these data demonstrated that BAR is a RING type E3 ubiquitin ligase. To elucidate target proteins of BAR E3 ubiquitin ligase will provide invaluable insights into the regulatory function of this ER protein complex.

Bap31 regulates the stability of BAR, whereas BI-1 is regulated by BAR

In search of target proteins for BAR E3 ubiquitin ligase, we tested if any caspases or any known BAR associating proteins could be target proteins (Figure 8). When coexpressed in 293T cells, none of the protein level of caspase 8, 9, or 10 was altered by BAR. Protein level of Bap31, Bcl-2, or Bap31-p20 was not changed by coexpression of BAR, either. However, the protein level of BI-1 was specifically decreased when cotransfected with BAR, but not BARdR (Figure 9). This effect was nullified by treatment of MG132, suggesting that reduced BI-1 level caused by BAR is through a proteasome dependent degradation pathway rather than due to difference in transfection efficiency. To further confirm that the transfection efficiency is the same, we will need to do quantitative PCR to confirm the same mRNA level under different cotransfection scenarios.

Although BAR does not change the protein level of Bap31, Bap31 functions to regulate the stability of BAR (Figure 10). Bap31 stabilizes the expression of BAR by decreasing proteasome-dependent degradation. Coexpression of Bap31 with BAR-full or BARdR shows that only BAR-full is stabilized by Bap31 (Figure 10 A). The stabilization of BAR is specific for Bap31 since the other two associating protein Bcl-2 or BI-1 has no effect on BAR expression (Figure 10 B). The ability of Bap31 to stabilize the protein level of BAR is negated by treatment of MG132, suggesting that Bap31 stabilizes the protein level of BAR by decreasing proteasome-dependent degradation rather than altering transfection efficiency (Figure 10 C). Using quantitative PCR, we showed that the mRNA level

of BAR is very similar with or without Bap31 coexpression (Figure 11), further confirming that Bap31 cotransfection does not change the transfection efficiency of BAR. Bap31 was shown to increase stability of all the BAR mutants with an intact RING domain, and full length of Bap31 is required to maintain the ability to protect BAR from degradation (Figure 12). There seems to exist a hierarchy of regulation that Bap31 regulates the protein level of BAR, which, in turn, regulates the protein level of BI-1.

One interesting observation is worth of mentioning. It is clear from our studies that RING domain plays a critical role in the regulation of protein level of BAR. However, the DED domain is somehow involved in the regulation of BAR stability as well. Mutation of four homologous critical hydrophobic Leu/Ile residues to alanines in the DED domain of BARdR, as has done with Bap31, makes a mutant named as BARdRmt. Even though BARdR was not sensitive to treatment of MG132, BARdRmt was expressed at much a lower level than that of BARdR and highly sensitive to treatment of MG132 (Figure 13), suggesting that DED domain of BAR may be involved in the interaction with proteins in ubiquitin-dependent degradation pathway as well.

Key Research Accomplishments

- BI-1 (Bax inhibitor 1), BAR (Bax inhibitor 2), Bap31, and Bcl-2 form a multi-protein complex at endoplasmic reticulum.
- Multiple domains was discovered to be involved in Bap31 homo-oligomerization and the interaction of BAR and Bap31.
- The RING domain of BAR was demonstrated to be essential for its E3 ubiquitin ligase activity, and is important for regulating the ubiquitination of itself.
- A hierarchy of regulation was revealed that Bap31 regulates the protein level of BAR, which, in turn, regulates the protein level of BI-1.

Reportable Outcomes

Not yet.

Conclusion

See “Key Research Accomplishment”.

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Appendices

Figures 1 to 13.

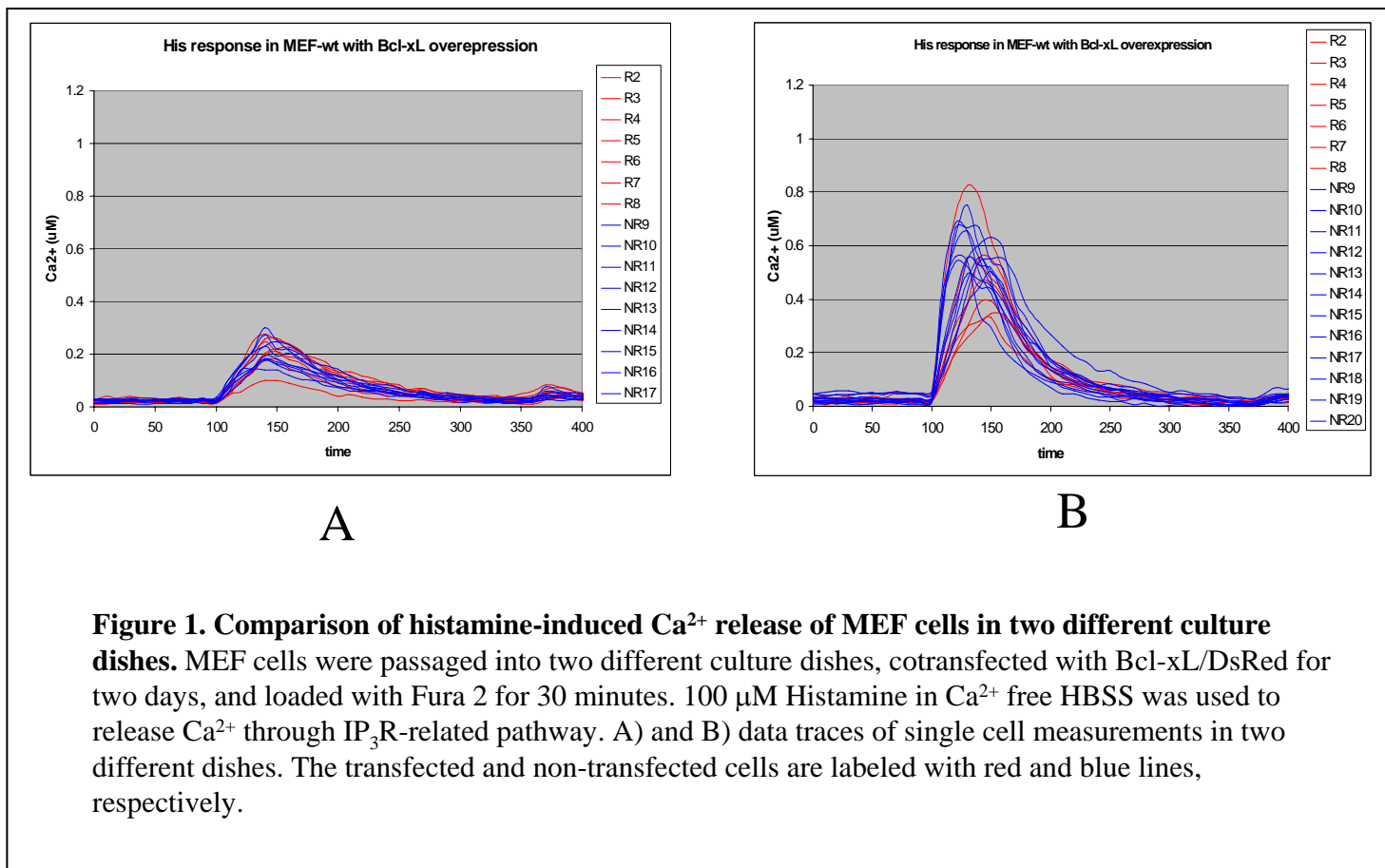
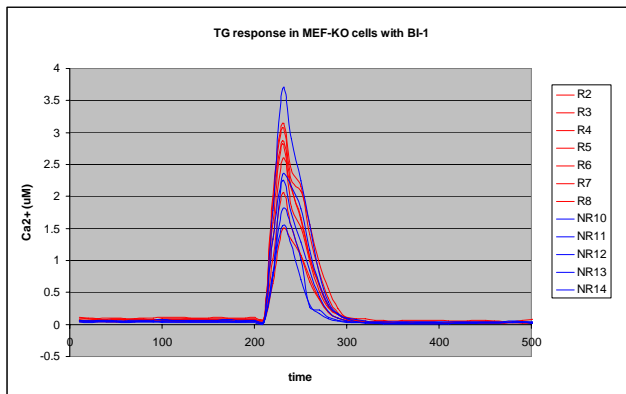
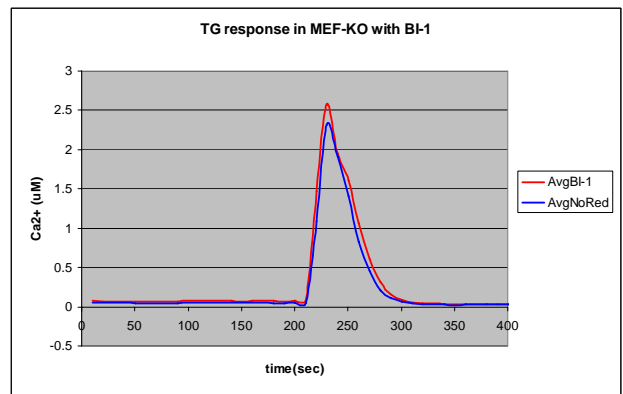


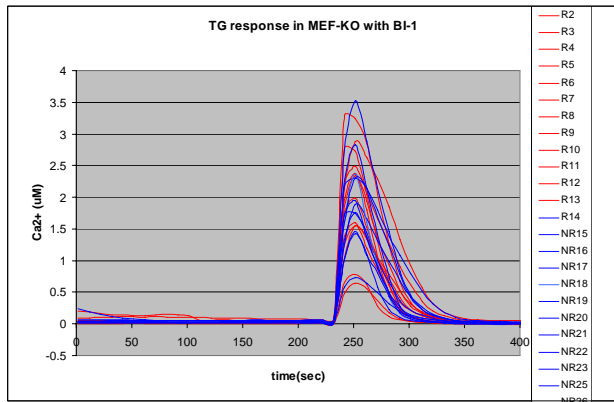
Figure 1. Comparison of histamine-induced Ca^{2+} release of MEF cells in two different culture dishes. MEF cells were passaged into two different culture dishes, cotransfected with Bcl-xL/DsRed for two days, and loaded with Fura 2 for 30 minutes. 100 μM Histamine in Ca^{2+} free HBSS was used to release Ca^{2+} through IP_3R -related pathway. A) and B) data traces of single cell measurements in two different dishes. The transfected and non-transfected cells are labeled with red and blue lines, respectively.



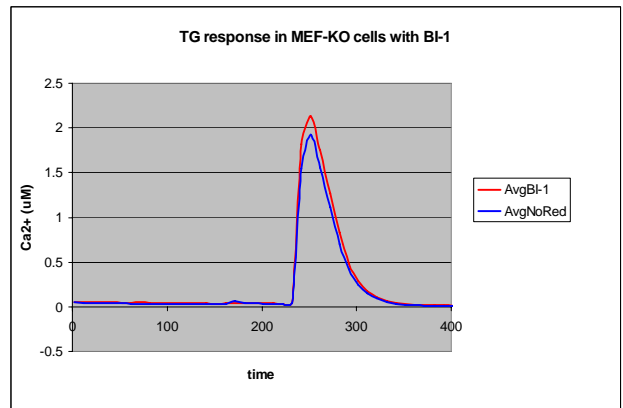
A



B

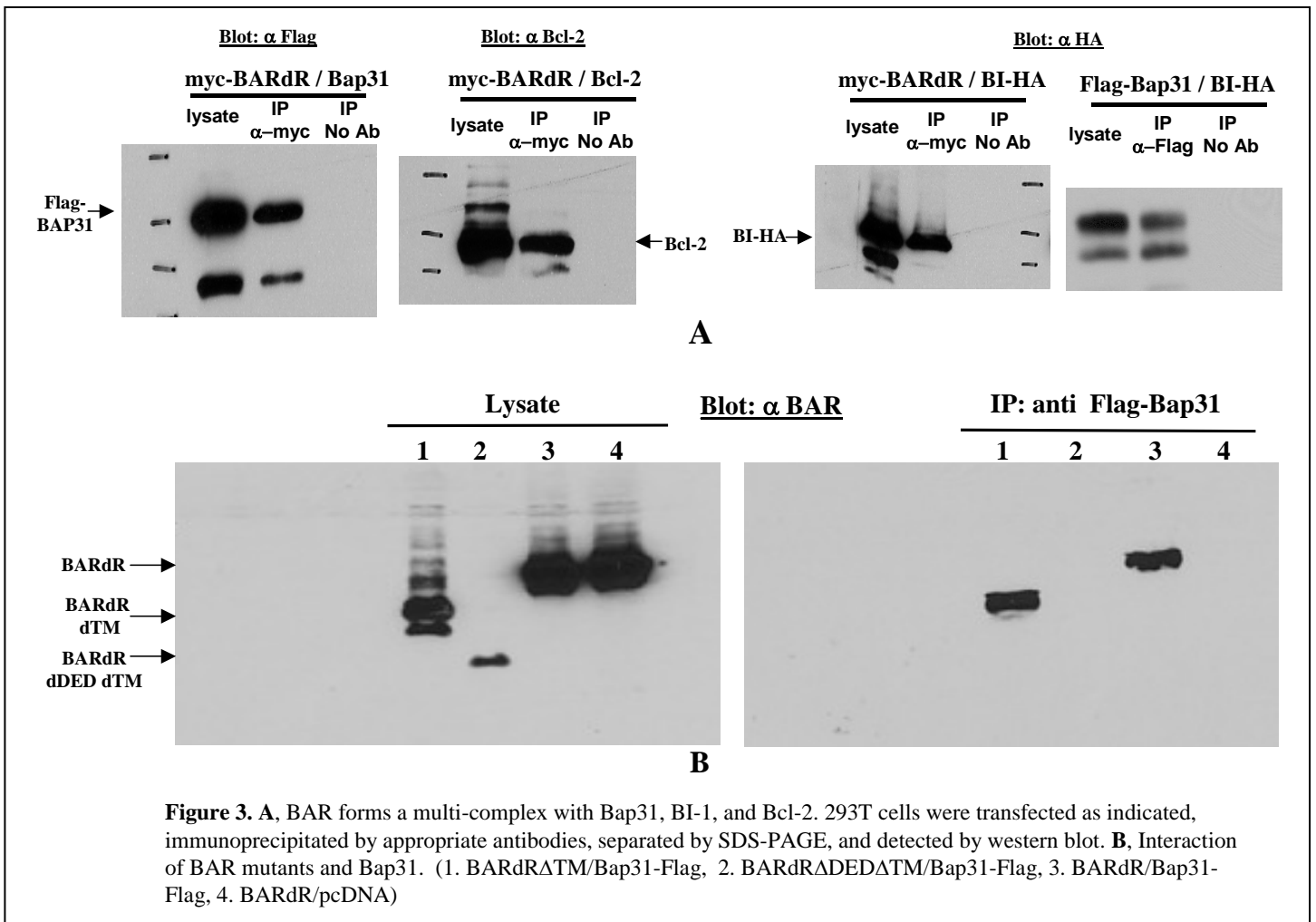


C



D

Figure 2. TG-induced Ca^{2+} release in BI-1^{-/-} cells transfected with BI-1. 1 μM TG in Ca^{2+} free HBSS was used to release Ca^{2+} from endoplasmic reticulum of MEF knockout cells transfected with BI-1. A) data traces of single cell measurements in one dish; B) average data of the single cell measurements in A). C) data traces of single cell measurements in another dish; D) average data of the single cell measurements in C). The transfected and non-transfected cells are labeled with red and blue lines, respectively.



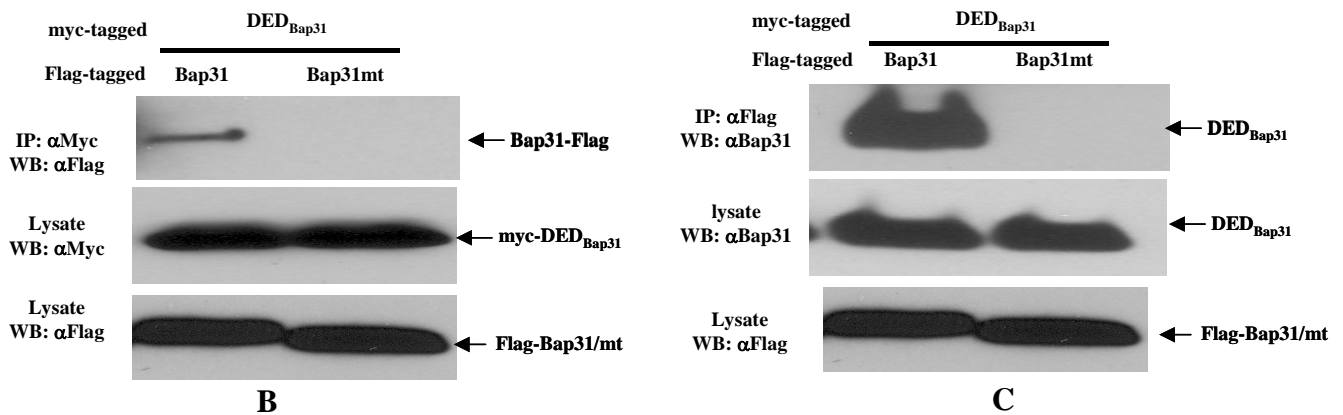
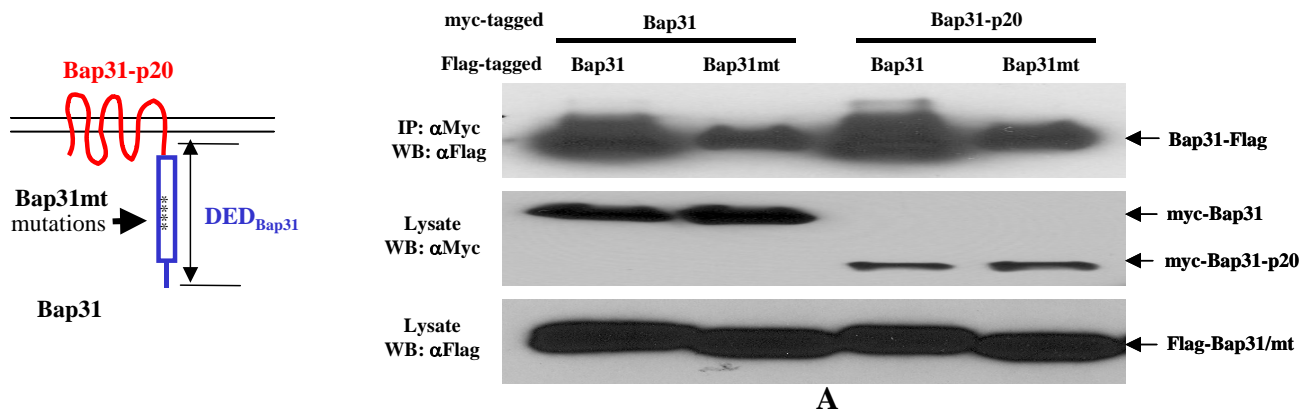
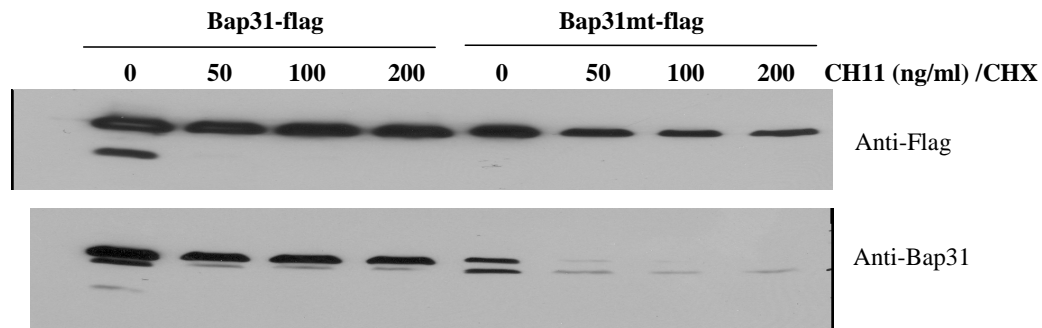
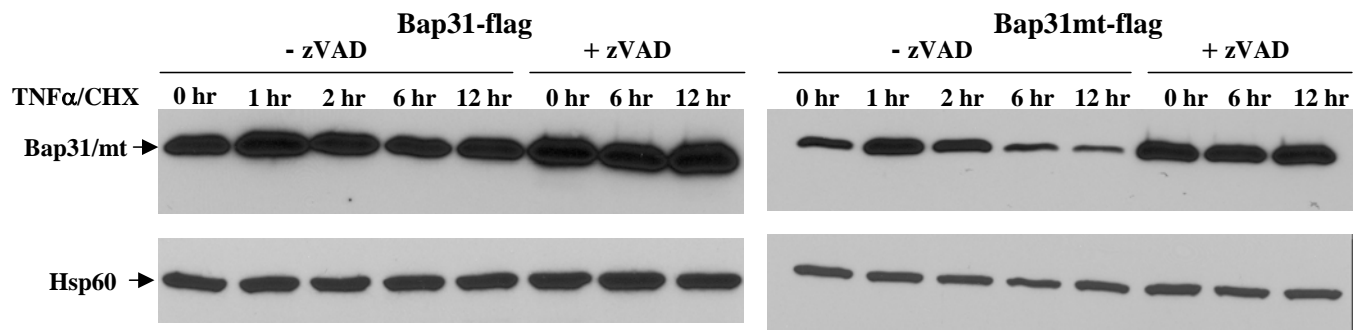


Figure 4. Both TM domain and pDED domain of Bap31 are involved in the homo-dimerization of Bap31. Both Bap31-p20 (mainly TM domain) and DED_{Bap31} retains ability to bind Bap31. Mutations in pDED domain (Bap31mt) has minimal effect on the binding of Bap31-p20 to Bap31 or its homo-dimerization (A). Mutations in pDED abolishes the binding of DED_{Bap31} to Bap31mt (B, C). These data showed that mutations in pDED specifically weakened the interactions between pDED domains, but has minimal effect on the interaction of other domains.

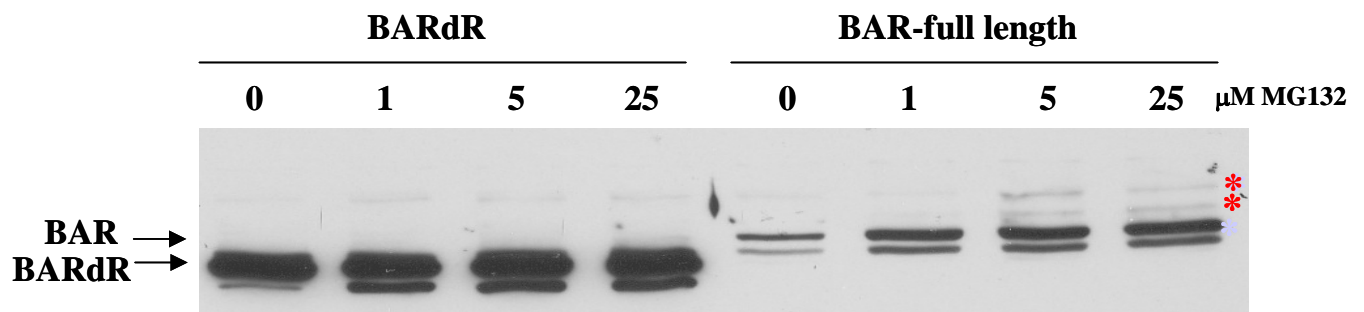


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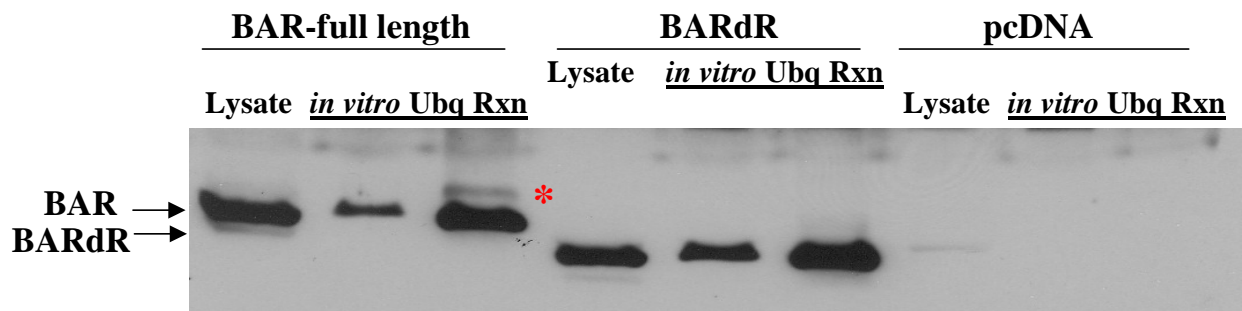


B

Figure 5, mutations in pDED domain of Bap31 render it more sensitive to caspase cleavage. A, HeLa cells transfected with Bap31 or Bap31mt were treated with 1 μ M CHX together with (0, 50, 100, 200 ng/ml) CH11 antibody for 16 hours. B, HeLa Cells transfected with Bap31 or Bap31mt were first treated with 1 μ M CHX for 30 minute, was then added 50 ng/ml TNF α for 0, 1hr, 2hr, 6hr, or 12hr. Caspase inhibitor, zVAD (50 μ M) were added to cells as indicated.



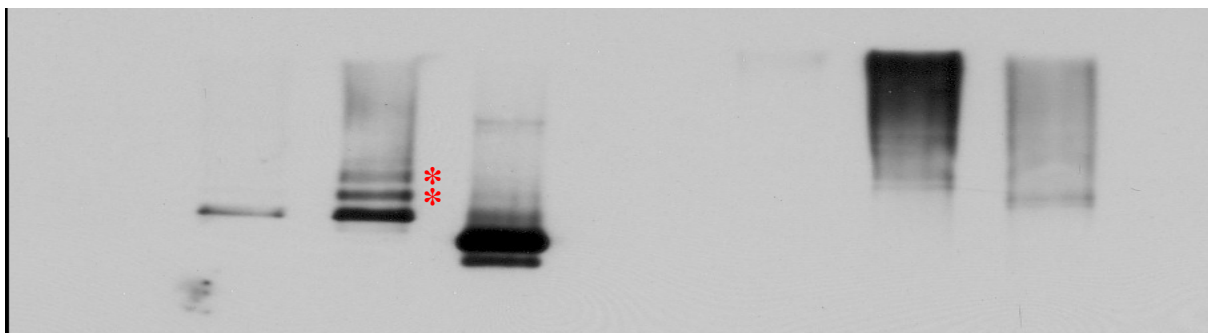
A



B

Figure 6. The RING domain is essential for ubiquitination and stability of BAR. A) 293T cells transfected with BARdR (RING domain deletion) or BAR-full length were incubated with MG132 (0, 1, 5, 25 μM) for 6 hours. Only BAR-full was significantly stabilized by MG132. B) 293T cells were cotransfected with Bap31 and myc-BARdR or myc-BAR-full. BARdR or BAR-full was pulled down by anti-myc antibody and protein G beads. The pulled down beads were incubated with *in vitro* Ubiquitination reaction mixture, separated by SDS-PAGE, and detected by anti-BAR antibody. Only BAR-full showed weak but clearly detectable ubiquitination product (labeled by a star).

Myc-BAR	+	+	-	+	+	-
Myc-BARdR	-	-	+	-	-	+
MG132	-	+	+	-	+	+
Ub-HA	+	+	+	+	+	+



IP: α myc

WB: α BAR

WB: α HA

Figure 7. The RING domain is essential for BAR's E3 ligase activity. 293T cells were cotransfected with Ubiquitin-HA and myc-BAR or myc-BARdR. The cells were then treated with or without 25 μ M MG132 for 6 hours. Cell lysate were immunoprecipitated with anti-myc antibody and protein G beads, which was separated by SDS-PAGE, and detected by anti-BAR and anti-HA antibody, respectively.

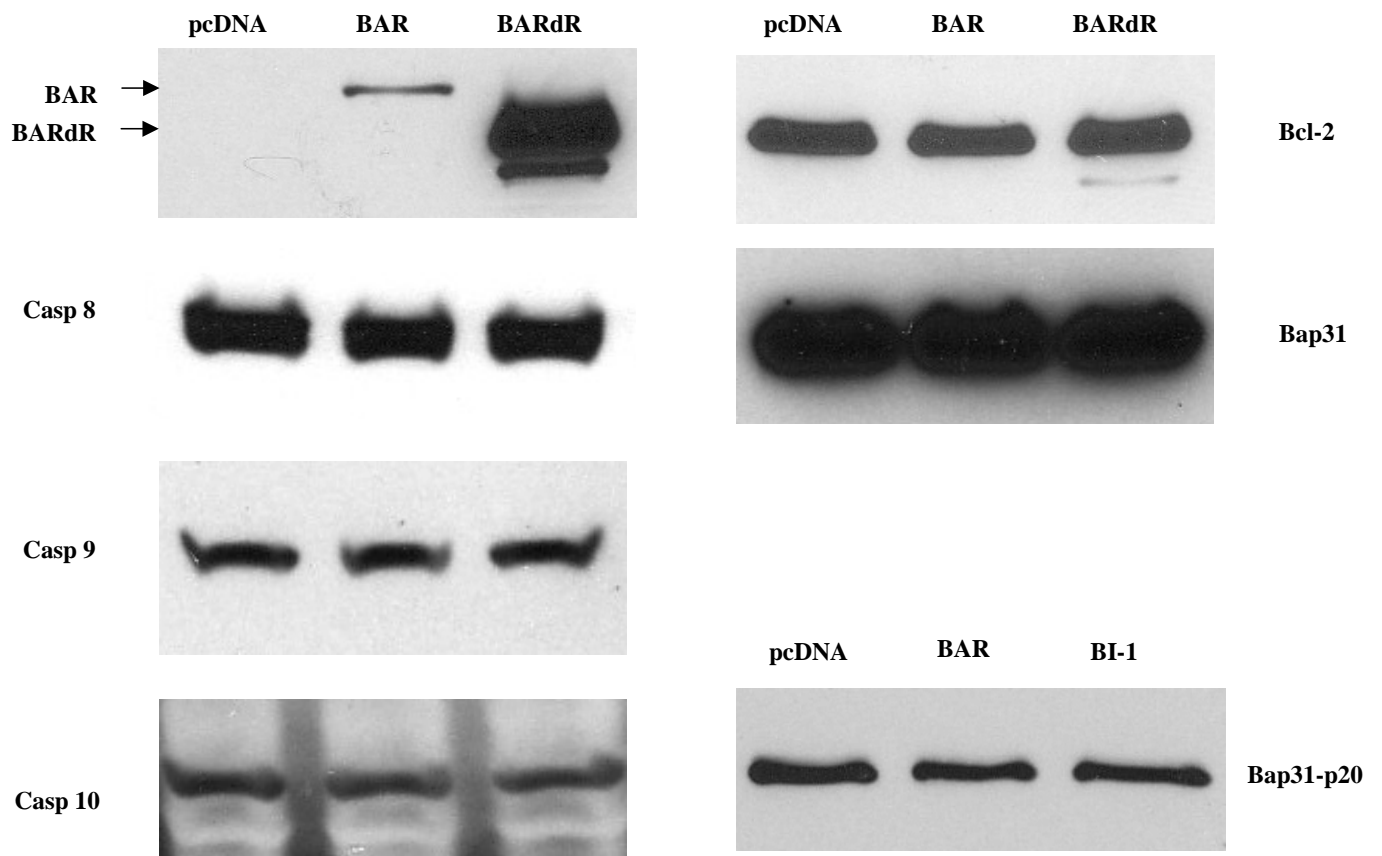


Figure 8. BAR was not shown to alter the protein level of selected protein targets. BAR full length, BARdR, or pcDNA was cotransfected with potential target proteins in HEK293 T cells. None of them was shown to have altered protein level when coexpressed with BAR-full.

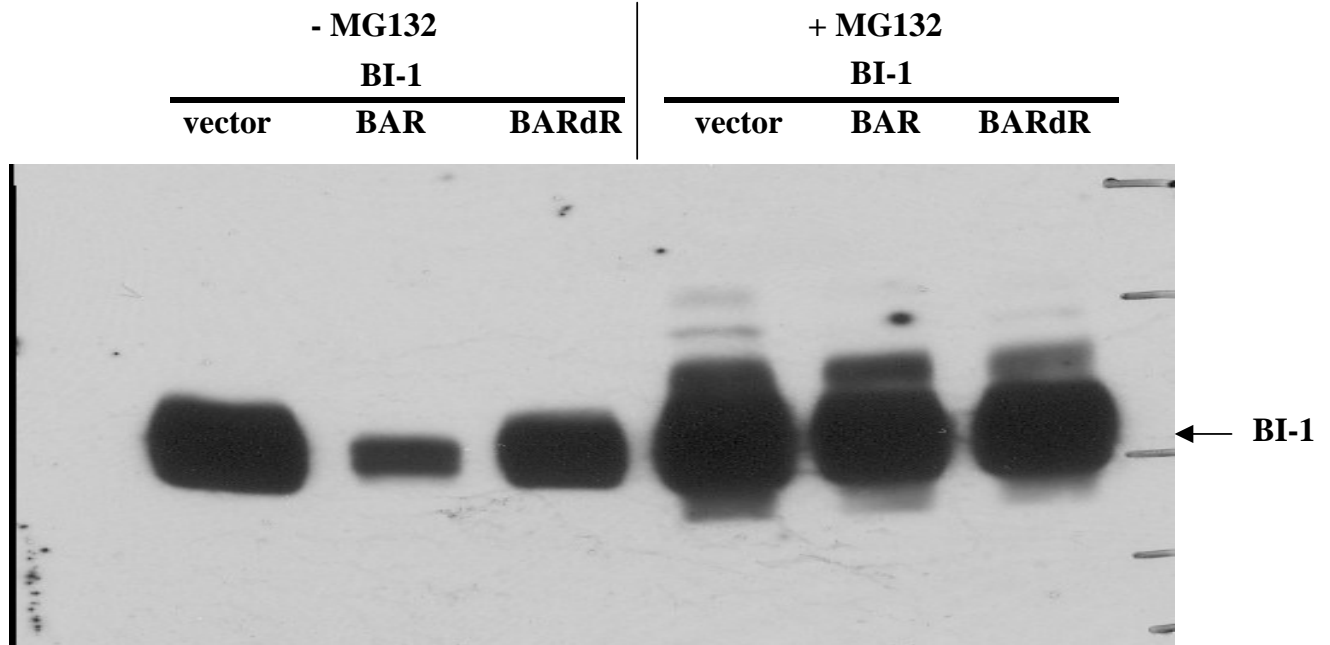
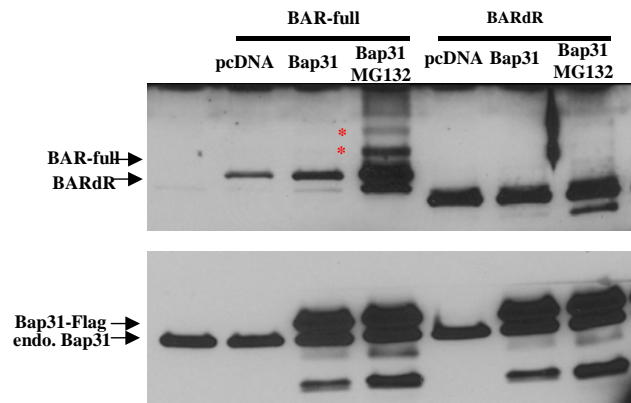
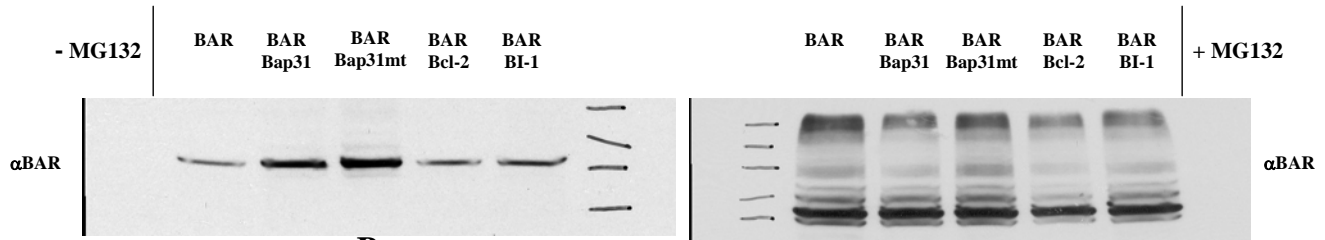


Figure 9, BAR, not BARdR, decreases the protein level of BI-1 when cotransfected in 293T cells. BI-1 was cotransfected with empty pcDNA3 vector, BAR full-length, or BARdR in 293T cells for 24 hours, and the transfected cells were treated with or without 25 μ M MG132 for 6 hours. Cell lysates were collected in lysis buffer, separated by 12% SDS-PAGE, and detected by western blot.



A



B

C

Figure 10. Bap31 stabilizes the expression of BAR by decreasing proteasome-dependent degradation. A) BAR-full, but not BARdR, forms multi-ubiquitinated product in the presence of MG132. Coexpression of Bap31 with BAR-full or BARdR shows that only BAR-full is stabilized by Bap31. B) only Bap31 or Bap31mt, but not Bcl-2 or BI-1, can stabilize the expression of BAR. C) The ability of Bap31 to stabilize the protein level of BAR is negated by treatment of MG132.

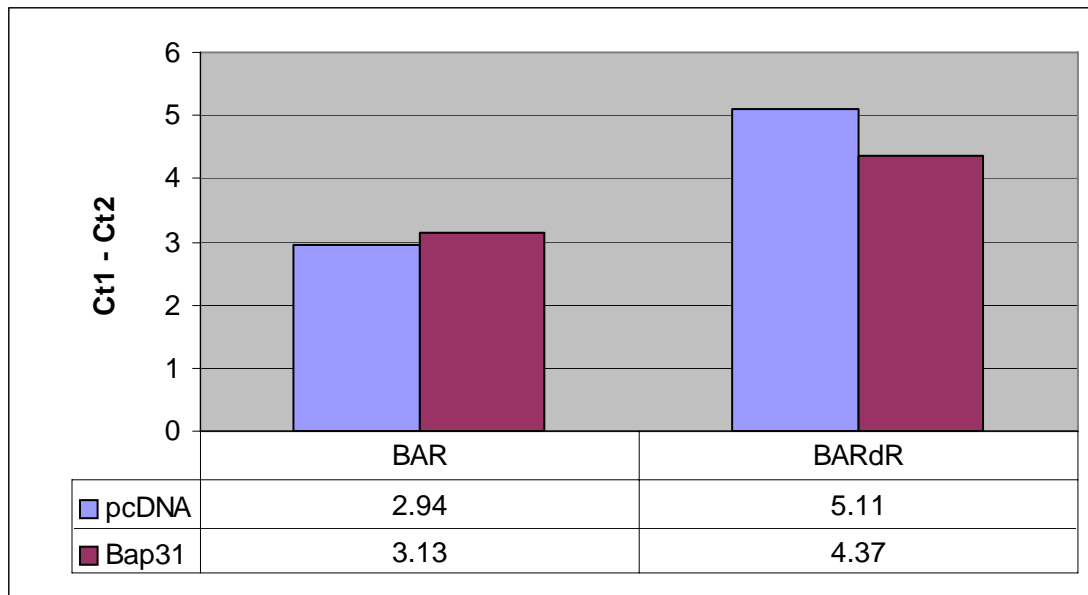
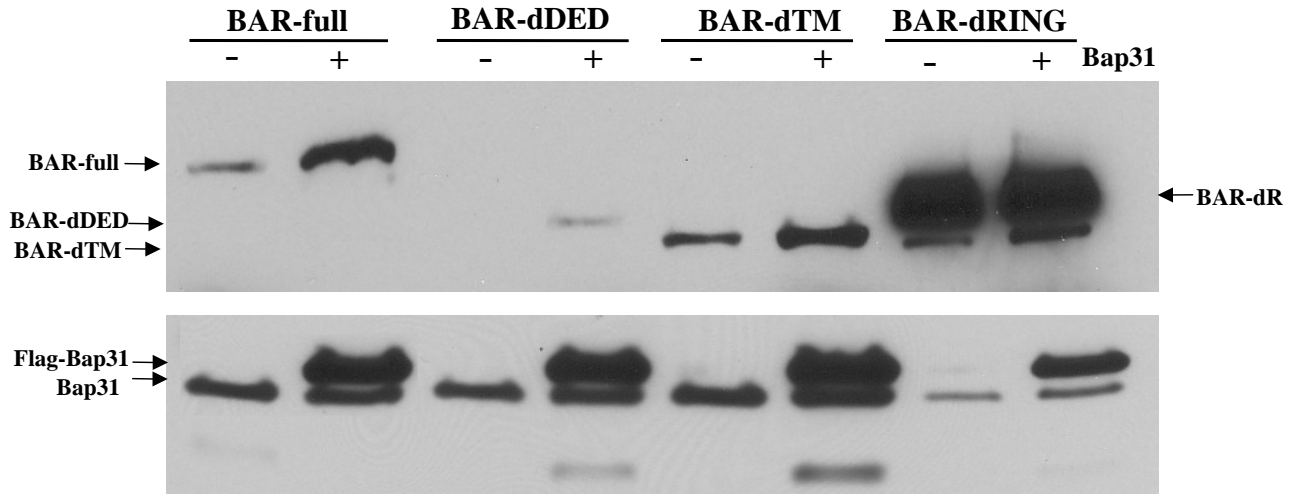
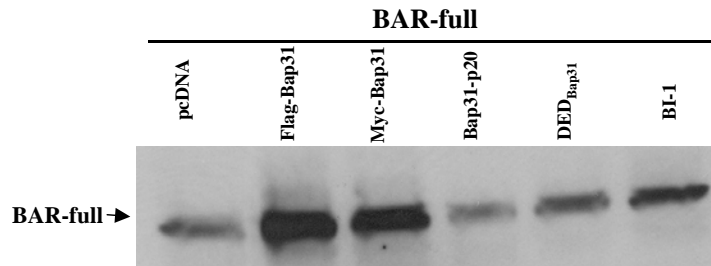


Figure 11, QPCR measurement of mRNA of BAR or BARdR in the presence or absence of Bap31. 293T cells were transfected with BAR or BARdR together with pcDNA or Bap31. Total RNA was extracted from transfected cells and converted to cDNA by reverse transcriptase. The relative amount of BAR or BARdR cDNA can be calculated from the difference of cycle number (Ct1-Ct2) between standard gene and target gene.



A



B

Figure 12. A, Bap31 stabilizes BAR mutants without pDED or TM domain. Different BAR mutations was cotransfected with pcDNA3 or Bap31 in 293T cells. Each lane was loaded with 50 μ g protein except for cell lysates from BAR-dRING, which was loaded with only 10 μ g protein. B, Full-length of Bap31 is required to protect BAR from degradation. Neither the N-terminal nor C-terminal of Bap31 is sufficient to stabilize the protein level of BAR.

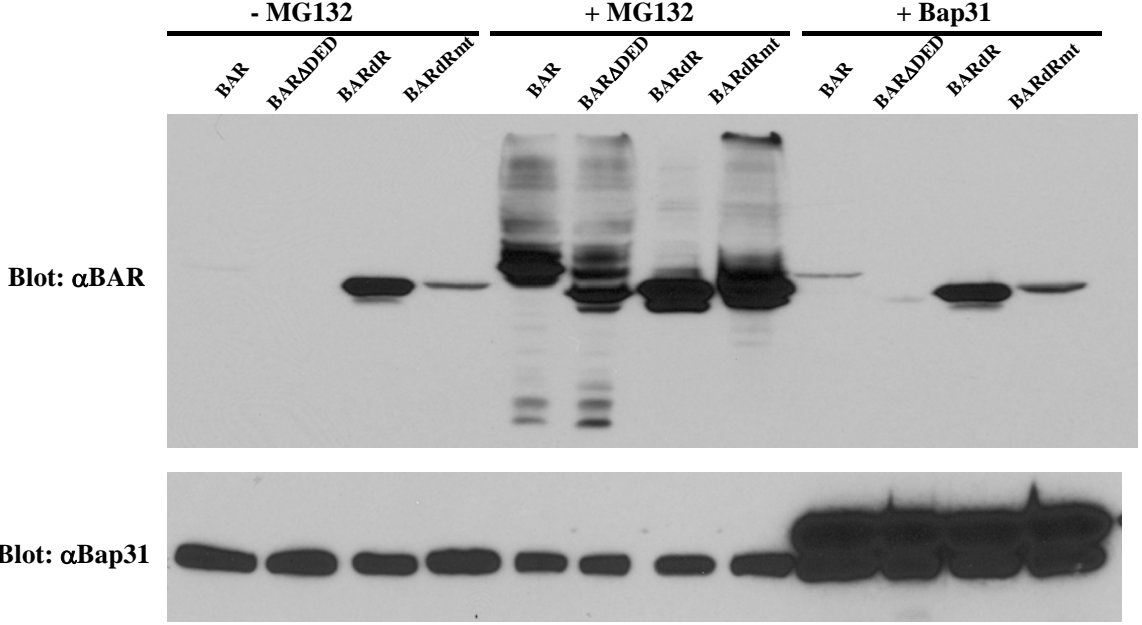


Figure 13. pDED domain of BAR may be involved in regulating the protein stability in a proteasome-dependent pathway. HEK293T cells were transfected with BAR, BAR Δ DED, BARdR, BARdRmt with or without Bap31. Cells were treated with 25 μ M MG132 for 12 hours compared with the non-treated ones.