Bid as a novel interacting partner of IRE1 differentially regulating its RNAse activity

Samirul Bashir[1] and Khalid Majid Fazili[1*]

[1]Department of Biotechnology, University of Kashmir, Hazratbal J&K, India

* Correspondence: fazili@kashmiruniversity.ac.in

Abstract

Unfolded protein response is a dynamic signalling pathway that is involved in maintenance of proteostasis and cellular homeostasis. IRE1, a transmembrane signaling protein that represents the start point of one of the UPR signaling cascades, which is highly conserved. IRE1 is endowed with kinase and endoribonuclease activities. Activation of Kinase domain of IRE1 by trans-autophosphorylation leads to activation of its RNAse domain. RNAse domain performs atypical splicing of Xbp1 mRNA and degradation of mRNAs by an effector function known as Regulated IRE1 Dependent Decay (RIDD). The regulation of distinctive nature of IRE1 ribonuclease function is possibly mediated by a dynamic protein structure UPRosome, which is an assembly of huge number of proteins on IRE1. Here, we reported that Bid is a novel recruit to UPRosome, which modulates IRE1 activity. Bid physically interacts with IRE1 and controls its phosphorylation levels in a negative manner. Bid overexpression displayed reduced phosphorylation levels of IRE1 and JNK, a downstream target of IRE1. Bid knockdown cells showed slightly enhanced IRE1 phosphorylation along with phosphorylation of JNK. Bid stimulated differential RNAse activity of IRE1 towards Xbp1 splicing and RIDD. These results establish that Bid is a part of UPRosome that differentially regulates IRE1 activation.

Introduction

ER provides the proper microenvironment and the necessary tools to accurately fold proteins (Helenius *et al*., 2004). Owing to varied physiological and pathophysiological conditions, the protein folding capacity of ER gets compromised, consequently leading to ER stress. In order to combat ER stress, cells have developed an ER to nucleus transcriptional network known as unfolded protein response (UPR). In mammalian cells UPR is composed of three main signaling branches namely IRE1 (Inositol requiring enzyme 1 signaling branch), PERK (PKR-like ER kinase) and ATF6 (Activating transcription factor 6) (Schroder *et al*., 2005; Walter *et al*., 2011). While in yeast UPR is mainly operated through IRE1 branch. The net initial effect of UPR is to reduce ER stress by increasing the amount of ER-resident chaperons, ER luminal

space, and other folding catalysts. However, if UPR fails to restore the homeostasis, it then initiates apoptosis in the cells (Jager *et al*., 2012).

The most conserved branch of the UPR signaling from yeast to humans is represented by IRE1 signalling network. IRE1 is a type I transmembrane protein with dual enzyme activity (kinase and endoribonuclease) localized to the ER membrane. Under non-stressed conditions IRE1 remains inactive through Bip binding but this inhibition is removed by induction of ER stress. Once activated, IRE1 performs splicing of Hac1 mRNA in yeast and Xbp1 mRNA in humans (Sidrauski *et al*., 1997; Gonzalez *et al.,* 1999; Calfon et al., 2002). Additionally, RNAse domain of the IRE1 is involved in the degradation of a subset of mRNAs through a process referred as regulated IRE1 Dependent Decay (RIDD) (Hollien *et al*., 2006; Hollien *et al*., 2009). The nature of Xbp1 splicing and RIDD activity is distinct. Xbp1 splicing is commonly linked to the cellular homeostasis whereas RIDD activity displays functional duality, where it can be cytoprotective or apoptotic depending on the stress conditions ER is facing. Though Xbp1 mRNA and RIDD substrates contain similar type of RNA architecture that is responsible for recognition and cleavage by IRE1 but the discrepancy between these two activities is not clearly understood. Therefore, it is plausible to uncover the mechanistic details involved in IRE1 activation and function.

The dynamics of IRE1 signalling is regulated by association of several protein factors. IRE1 is thought to assemble in a huge protein complex designated as UPRosome (Hetz *et al*., 2009), where it is thought to act centrally and interact with a number of proteins which can either activate or inactivate it or connect IRE1 with other signalling networks. UPRosome as a multimeric protein complex has a great potential to explain the mechanistic details of IRE1 activation and its downstream effector functions in particularly for understanding the dual nature of IRE1 activity. Several members of Bcl-2 protein family have been found to interact with IRE1 and modulate its activity. Binding of Bak and Bax proteins to IRE1 stabilize the oligomer and maintain its activation. Additionally, the presence of Bak and Bax effected IRE1 mediated JNK activation (Hetz *et al*., 2006).

The members of Bcl-2 protein family interact with each other, therefore it is possible that other members of the family are recruited to UPRosome and regulate IRE1 dynamics. In this context, we screened interactions between IRE1 and the three proteins members from the Bcl-2 family namely Bid, Bik and Bcl-2. We found that Bid directly interacts with IRE1 and modulates its activation. We observed that Bid interaction to IRE1 decrease its trans-autophosphorylation activity. We also observed that Bid specifically impacts IRE1 signalling and decreases the

phosphorylation levels of IRE1 and its downstream target JNK. Interestingly, our results revealed that Bid differentially regulates two outputs of IRE1 RNAse activity, Xbp1 splicing and RIDD. Bid repressed IRE1 is able to perform Xbp1 splicing but loses its RIDD activity. Together, our results added another regulatory element to UPRosome and are suggestive of an atypically novel function of Bid protein in ER stress induced IRE1 signalling pathway.

Results

Bid physically interacts with IRE1

To identify the interactions between IRE1 and Bcl2 family of proteins, immuno-precipitation assay was performed. Full length Ire1 (untagged) was co-transfected with GST-tagged Bid, GST-tagged Bik, GST-tagged Bcl-2 and GST-tagged vector independently in HEK293T cells. This was followed by tunicamycin treatment to induce ER stress for 6hr. For immunoprecipitation (IP) assays, we selected time points that correspond to the later stages of ER stress, because we thought that Bcl-2 proteins might be recruited to UPRosome at this stage. Initially we performed IP at eight hours post tunicamycin treatment, however no interaction was observed at this stage. Next, we performed IP at six-hour post tunicamycin treatment and interaction was observed. Results revealed the presence of a band corresponding to Gst-Bid in the blot, while no bands were present for Bik and Bcl-2 IP. Also, there was no band present in Mock and Vector control, demonstrating the specificity of the assay (Figure 1A). Since intensity of Gst-Bid band was less, therefore we repeated IP assay for Bid and Bik (Figure 1B), where we could see an intense band present in Bid IP, while Bik IP didn't show any band. Blots were also probed with anti-IRE1 antibody to detect the presence of IRE1 in pulldown samples (Figure 1A and B). Then we performed reverse IP, where we did pulldown using anti-Gst antibody and western blot was probed with anti-IRE1 antibody (Figure 1C). In this experiment, we observed a band corresponding to IRE1 in Bid pulldown assay only, while no such band was observed in Bik and controls. Hence, we conclude that among the Bcl-2 proteins, only Bid was found in a complex with IRE1.

In order to identify the nature of interaction between IRE1 and Bid, we performed yeast two hybrid assay. In this experiment IRE1 was translationally fused with GAL4 activation domain (GAL4AD), while Bid was fused with the GAL4 DNA binding domain (GAL4BD). We used AH109 strain of *Saccharomyces cerevisiae* that harbours a chromosomally integrated His (Histidine) reporter gene integrated chromosomally. Colonies obtained from co-transfection of

IRE1-GAL4AD and Bid-GAL4BD were grown in SDL$^-$T$^-$broth and a series of dilution was prepared and then spotted on SDL$^-$T$^-$H$^-$ plates. Similarly, spotting was done on YPD to check the viability of cells (Figure 1D). We observed that IRE1-GAL4AD and Bid-GAL44BD co-transformed colonies grew up to 5th dilution (1:100000), while controls showed highly restricted growth (Figure 1D). This implies that in IRE1+Bid co-transfected colonies, the GAL4 transcription factor was successfully reconstituted by interaction between IRE1 and Bid, which lead to expression of His-reporter gene and allowed growth on His drop out media. Therefore, we conclude that Bid protein shows a direct physical interaction with IRE1.

Bid negatively regulates phosphorylation of IRE1α and JNK

To demonstrate the effect of Bid protein on the phosphorylation levels of IRE1, we performed both Bid gain of function and loss of function studies. Cells were transiently transfected for Bid, vector and Mock followed by treatment with 6μM tunicamycin for six hours. For Bid depleted conditions, cells were transfected with Bid siRNA along with a negative control siRNA followed by tunicamycin treatment. Our results revealed that Bid overexpression drastically decreased the levels of pIRE1 compared to vector control and Mock (Figure 2A). Interestingly, our results also revealed that the levels of IRE1 phosphorylation increase in Bid knockdown conditions compared to controls (Figure 2B). These results therefore concluded that Bid negatively regulates IRE1 phosphorylation.

A number of studies have revealed that activation of IRE1 upon ER stress is associated with the activation of JNK (Urano *et al*., 2000; Yoneda *et al*., 2001). Since our experiments revealed a negative regulation of IRE1 phosphorylation driven by Bid. We decided to investigate the effect of Bid on phosphorylation/ activation of JNK. Similar sets of experiments were designed to check the effect of Bid on JNK phosphorylation. In consistent with IRE1 phosphorylation status, we observed that there is a decline in JNK phosphorylation upon Bid overexpression compared to Vector and Mock (Figure 2C). Similarly, JNK phosphorylation was enhanced in Bid depleted conditions compared to negative siRNA control and Mock (Figure 2D). Together these results suggest that Bid modulates JNK phosphorylation in a similar fashion to IRE1.

Bid differentially modulates IRE1-RNAse outputs

The most comprehensive documented function of IRE1 is performed by its RNAse domain that catalyses the splicing of Xbp1 mRNA by excising a 26-nucleotide intron. IRE1 phosphorylation is important for catalysis of Xbp1 splicing and has been shown that for successfully Xbp1 splicing, phosphorylation of kinase activation loop becomes indispensable. This was further supported by an observation where unmitigated splicing of Xbp1 was detected in a phosphomimetic mutant of yeast IRE1. In our experiments, we found a decline in IRE1 phosphorylation levels in presence of overexpressed Bid protein. To evaluate the effect of Bid gain of function and loss of function on the splicing of Xbp1, we analysed the levels of spliced XBP1 protein. Surprisingly, we found that the levels of Xbp1 remained unchanged in both overexpression and knock down experimental conditions (Figure 3A and B). Although the levels of pIRE1 changed in response Bid presence, but we didn't find any change in spliced Xbp1 levels. We also checked the splicing status of Xbp1 by semi-quantitative RT-PCR. Because of the presence of 26-nucleotide intron, unspliced Xbp1 (Xbp1u) runs slower than the spliced mRNA product. Here also we didn't find any change in Xbp1 splicing status (Figure 3 C). These results pointed towards the fact that Xbp1 splicing operates even under low IRE1 phosphorylation states.

The RNAse domain of IRE1 is also involved in degradation of mRNAs preferably localised to ER through RIDD. Although the substrates of RIDD share structural similarities with Xbp1 mRNA, however IRE1 shows differential activity towards Xbp1 splicing and RIDD. But RIDD activity is also dependent on phosphorylation status of IRE1. So, we wanted to analyse the effect of Bid gain of function and loss of function on RIDD activity. For evaluation of RIDD activity, we analysed the levels of scara3 (a known RIDD target) by real time PCR analysis of scara3 mRNA. Interestingly it was found that scara3 mRNA levels elevated in Bid overexpression compared to controls, Vector and Mock that illustrates reduced RIDD activity in Bid overexpressing conditions (Figure 3D). We also observed slightly improved RIDD activity upon Bid knockdown (Figure 3E). These results demonstrated that RIDD activity is severely defected in Bid overexpression state that is in consistent with decrease in IRE1 phosphorylation levels. Our results are in corroboration with a study, where they showed that modulating IRE1 phosphorylation/oligomerization displays differential Xbp1 splicing and RIDD outputs (Han et al., 2009). Taken together these results, we suggest that Bid differentially regulates IRE1 RNAse activity.

Discussion

IRE1 branch of the UPR signalling network represents the highly conserved pathway of all the three and it provides a major platform for deciding fate of the cells under stress. IRE1 harbours dual enzyme activity, kinase and endoribonuclease. Activated IRE1 catalyse the non-canonical splicing of Hac1 mRNA in yeast and Xbp1 mRNA in humans (Sidrauski *et al*., 1997; Gonzalez *et al*., 1999 and Calfon *et al*., 2002) and degrades a subset of mRNAs localized to ER through RIDD (Hollien *et al*., 2006; Hollien *et al*., 2009). The activation of IRE1 depends on the presence of positive and negative regulators. UPRosome was identified as a huge protein complex assembled at IRE1 that regulates the dynamics of UPR signalling pathway. Protein factors recruited to UPRosome either activates or deactivates IRE1 in a stress dependent manner (Hetz et al., 2009, Woehlbier et al., 2011). One of the interesting fact about UPRosome is that factors recruited to it act atypically and perform their functions different from their originally designated functions. Bak and Bax proteins have been found associated in a complex with IRE1, thereby activate its downstream target Xbp1 and promote cell survival (Hetz *et al*., 2009). To better understand and unravel the role of UPRosome in regulating activity of IRE1, which form the central core component of the UPRosome, we investigated the role of other Bcl-2 family members like Bid, Bik and Bcl-2. We found that Bid is novel interactor of IRE1 that modulates its activation and downstream signalling.

Bid is a BH3 only protein that upon cleavage of the C-terminal fragment (tBid) binds to Bak and Bax and promotes permeabilization of OMM and cytochrome C release. Like Bak and Bax, we found that Bid also interacts with IRE1 and modulates its activation. The interaction is direct in nature as was revealed by yeast two hybrid assay. Here, we provided evidences for negative regulation of IRE1 phosphorylation driven by Bid. We observed a transition in phosphorylation levels of IRE1 between different states of Bid. Overexpression conditions of Bid declined IRE1 phosphorylation drastically and improved IRE1 phosphorylation levels were observed in Bid depleted conditions. These two contrasting conditions substantiated the fact that Bid has a negative effect on IRE1 phosphorylation. Also, JNK phosphorylation levels were found decreased upon Bid overexpression and reverted back rather increased in Bid depleted conditions. JNK activation is linked to the phosphorylation of IRE1 and IRE1 has been associated with activation of JNK through IRE1-TRAF2 module. Consistent with IRE1 phosphorylation, JNK phosphorylation levels follows a similar trend in Bid overexpression and knockdown states. IRE1-JNK line has been linked to promote the cell death in response to chronic ER stress, thus places Bid at a

position to promote cell survival by disrupting IRE1-JNK activation. These results might partially explain the role of Bid in cell proliferation (Bai *et al*., 2005; Xiao-Ming *et al*., 2006). However, we need to further validate these results in a model organism and in detail investigate the cell survival role of Bid in response to ER stress.

One of the interesting findings of our results revealed that Bid differentially activates IRE1 RNase activity. It was found that overexpression of Bid severely inhibited degradation of RIDD target scara3. This effect was reversed in Bid knockdown conditions, where we observed slightly enhanced RIDD activity. In both the conditions, levels of spliced Xbp1 mRNA remained unchanged. This differential RNase activity is possibly linked to the phosphorylation state of IRE1. Our results are in corroboration with the previous studies where binding of 1NM-PP1 (an ATP analog) to kinase dead mutant I642G Ire1 rescued Xbp1 splicing partially but failed to activate the RIDD (Papa *et al*., 2003; Han *et al*., 2009). These studies also lead to the observations that ATP-competitive inhibitors can allosterically active RNAse domain of IRE1. In case of wild type IRE1, APY29 (type I kinase inhibitor) was found to decrease trans-autophosphorylation. This kind of inhibition limited RIDD activity but allowed efficient splicing Xbp1 (Han *et al*., 2009). IRE1 phosphorylation is directly associated with the formation of a higher order oligomeric structure. Averting the formation of oligomeric IRE1 would alter its ribonuclease function (Ghosh *et al*., 2014). It is thought that Xbp1 represents the most preferred substrate for IRE1 RNAse domain that is why even in lower order oligomeric structure it is being spliced. However, for RIDD activity higher oligomeric state of IRE1 is inevitable. Similarly, we think that Bid mediated suppression of IRE1 phosphorylation is responsible for preventing the generation of higher order IRE1 oligomer and allows only Xbp1 splicing to occur. We propose a model, where presence of Bid inhibits higher order oligomeric IRE1 structure formation and thus keeps RIDD under check, but fully supports Xbp1 splicing. However, to better understand this scenario, we have to monitor the effect on IRE1 oligomerization in presence and absence of the Bid.

Methods

Cell culture and treatments

HEK293-T cells were grown at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and 1 × penicillin-streptomycin solution. Cells were treated with 6µM tunicamycin for UPR induction.

Immuno-precipitation

For immunoprecipitation, HEK293-T cells were transfected with pcDNA3.1 (+) IRE1 and pEBG-Bid / or pEBG-Bik /or pEBG-Bcl2 clones using PEI transfection reagent. Post 48hr transfection cells were treated with 6µM tunicamycin and incubated for 6 hr. As a control cells transfected with pcDNA3.1 (+) IRE1 and pEBG empty vector and treated similarly. Cells without transfection were also treated with tunicamycin. Protein lysates were prepared using NETN buffer. 120µL of NETN buffer was added and incubate on ice for 1hr. Supernatant was collected after centrifugation at 14000rpm for 25min. Meanwhile, Protein G plus Agarose beads were washed three times with NETN buffer and incubated with 2µg of primary antibody (Anti- GST or IRE1α (14C10) Rabbit mAb) in 40µL of 1XTBS, overnight on a Nutator (rotating apparatus) at 4°C. On consecutive day protein lysate was added to the mixture and incubated on Nutator overnight at 4°C. On the third day of experiment bead solution was centrifuged at 2000rpm for 2min. Then washed thrice with NETN buffer by centrifugation. 30µL of 2X laemmili Loading Buffer were added to the samples and heated at 100ºC for 15min. Samples were then run on 12% SDS PAGE followed by western blotting using (IRE1α (14C10) Rabbit mAb or Anti- GST) antibody.

Western Blotting

40-50µg of protein lysates were run on 12% SDS PAGE and transferred PVDF membrane. Membrane was blocked in 3% blocking solution (3% BSA in 1XTBS) overnight. Blot were incubated overnight in specific primary antibody (1:1000 to 1: 2000 dilution) at 4ºC. Secondary antibody against the mouse or rabbit primary antibody was added and incubated at room temperature for 2hr. Depending on the nature of secondary antibody, detection assays were performed. In this study, we used two types of secondary antibodies. IRDye Goat anti-Rabbit IgG (1: 50000 dilution), Goat anti-Mouse IgG ((1: 50000 dilution) are fluorophore conjugates and visualized in different fluorescence channels, 800nm and 700nm respectively with the help of LicoR detection system (Odyssey). The other type of secondary antibody is an ALP

conjugate (1:40,000 dilution). The blot was kept in ALP developing solution for 2-3min until bands appear on the membrane. Then the blot was washed with tap water and analysed on Gel doc system.

Yeast two-hybrid assay

For the purpose of yeast two-hybrid assay, Ire1 and Bid genes were cloned in pGAD424 and pGBT9 yeast vectors respectively. pGAD424 harbours GAL4 activation domain and pGBT9, contains GAL4 transcription binding domain. Both the plasmids were co-transformed in AH109 yeast strain, which harbours a His-reporter gene. Single colonies were picked and patched on $SDL^{-}T^{-}$ Agar and incubated at 30°C for three days. Once colonies grew, they were spotted on $SDL^{-}T^{-}H^{-}$ (SD complete media with leucine, tryptophan and histidine drop out) plates. Plates were continuously observed for 5-6 days to assess the growth. Similarly, co-transformation of pGAD-Ire1 + pGBT9 vector and pGBT-Bid + pGAD4242 was done to set a negative control for the assay. Since histidine biosynthesis gene promoter is responsive to GAL4 transcription factor. Therefore, expression of His-gene will depend on the reconstitution of GAL4 transcription factor by direct interaction between Ire1 and Bid that would allow the growth of colonies on $SDL^{-}T^{-}H^{-}$ drop out media.

siRNA knockdown

HEK293-T cell line was transfected with Mission esiRNA human BID using Lipofectamine™ 2000 reagent. Mission siRNA fluorescent universal negative control #1, Cyanine 3 was also transfected, acting as a negative control for siRNA knock down. HEK293-T cells were seeded in five different 60mm dishes for siRNA transfection. Cells were taken for transfection at 65% confluency. Two different transfection mixtures A and B were prepared. Mixture A was prepared by mixing 1400ng of siRNA with 250µL of DMEM and incubated at room temperature for 5min. Mixture B was prepared by adding 5µL of lipofectamine to 245µL of DMEM. Mixture A and B was mixed and incubated for 20min at room temperature. Media was removed from the dishes, and 1.5mL of DMEM was dispensed followed by addition of transfection mixture and incubated in incubator at 37°C incubator. Post 12-16hr of incubation, media was removed and fresh 3mL of complete media was added. Cells were harvested at 24hr, 48hr and 72hr post siRNA transfection along with the negative control and mock (without transfection). Then samples were subjected to Western blotting to analyse the Bid knockdown by probing with Anti-Bid antibody.

RNA isolation, Reverse transcription and real-time PCR

Total RNA was extracted from the cells using Trizol. cDNA was synthesised from total RNA with M-MLV Reverse transcriptase using random primers. 2-5µg of RNA was used for each reverse transcription reaction. Real time PCR was performed with SYBRGreen fluorescent reagent to check the abundance of specific transcripts and for each reaction 75ng of cDNA was used. Actin or GAPDH primers were used as an endogenous control. Reaction was carried out in 7500 Real-Time PCR System (Applied Biosystems). By normalizing the comparative threshold cycle with endogenous controls, quantitation of each transcript was done.

Statistical analysis

The results presented in this study are expressed as means ± standard error. GraphPad PRISM v6.03 statistical software (GraphPad Software, La Jolla, CA) was used for statistical analysis. One-way ANOVA was used to determine the significance of differences between different groups. A p-value of 0.05 was considered significant.

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Figures

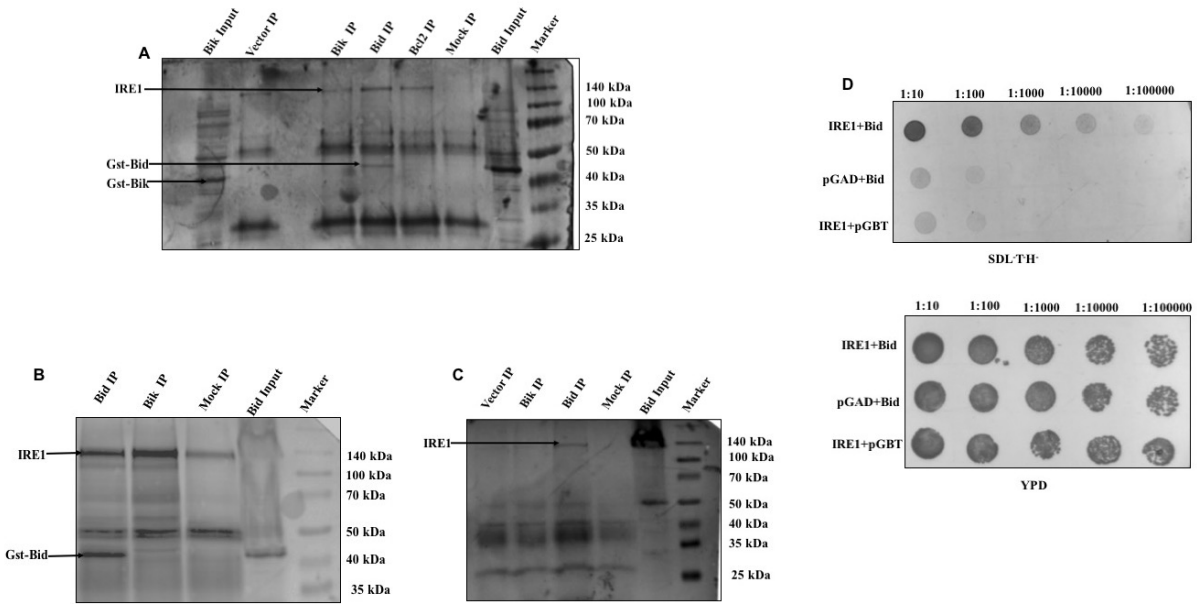

Figure1: Bid physically interacts with IRE1. A) HEK293-T cells were co-transfected with full length IRE1 and Gst-Bid, IRE1 and Gst-Bik, IRE1 and Gst-Bcl2 and IRE1 and Gst-Vector. Cells were treated 6μM tunicamycin for 6hr. Immunoprecipitation was performed with anti-IRE1 antibody. Presence of Bcl2 proteins in IRE1 complex was detected by western blot analysis using anti-Gst antibody. Bid IP, Bik IP, BCl-2 IP and Vector IP corresponds to immuno-pulldown experiments for Gst-Bid, Gst-Bik, Gst-Bcl2, and Gst-vector (pEBG) co-expressed with Ire1 respectively. Mock IP represents HEK293-T cells without transfection. Marker represents protein molecular mass indicator. B) Repeated Immuno-precipitation experiment for Bid and Bik. Blots were also probed with anti- IRE1 antibody to check presence of IRE1 in pulldown samples. C) Immuno-precipitation experiment for Bid and Bik with Gst-antibody. D) pGAD-Ire1 + pGBT-Bid represents colonies obtained from co-transfection of pGAD-IRE1 and pGBT-Bid plasmids. pGAD424 + pGBT-Bid shows colonies obtained from co-transfection of pGAD424 and pGBT-Bid plasmids. pGAD-IRE1 + pGBT9 represent colonies obtained from co-transfection of pGAD-IRE1 and pGBT9 plasmids. Dilution spotting on $SDL^{-}T^{-}H^{-}$ drop out media to select for positive interactors (Upper panel). Lower panel shows dilution spotting on YPD rich media. Dilutions were made up to 10^{-5}.

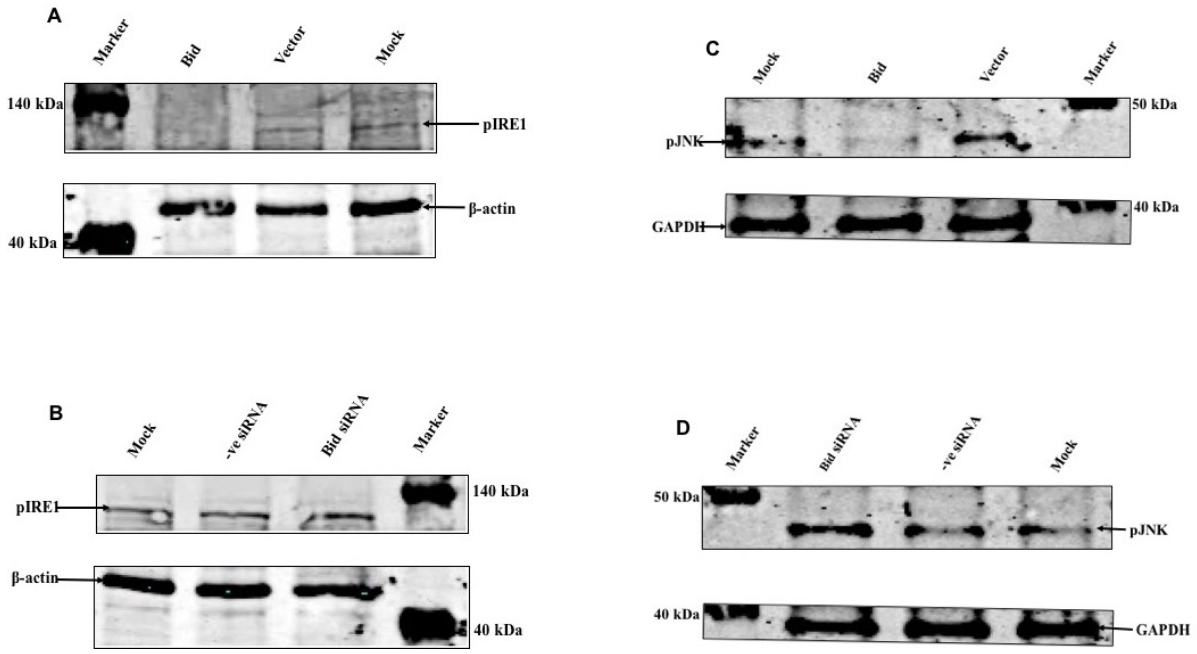

Figure2: Bid negatively regulates phosphorylation of IRE1 and JNK. A) Analysis of IRE1 phosphorylation in Bid overexpressing conditions. HEK293-T cells were transiently transfected for Bid, Vector and Mock. After 42hr cells were stimulated with 6µM tunicamycin for 6hr. Levels of pIRE1 were checked by probing with Anti-IRE1 (phosphoS724) antibody. B) Analysis of IRE1 phosphorylation in Bid knockdown conditions. HEK293-T cells were transfected with Bid siRNA and –ve siRNA followed by treatment with 6µM tunicamycin for 6hr. C) HEK293-T cells were transiently transfected for Bid, Vector and Mock. After 42hr cells were stimulated with 6µM tunicamycin for 6hr. D) HEK293T cells were transfected with Bid siRNA and –ve siRNA followed by treatment with 6µM tunicamycin for 6hr. Western blot analysis was performed to check the levels of pJNK by probing with Phospho-JNK Rabbit mAb. β-Actin and GAPDH represent the endogenous controls for pIRE1 and pJNK respectively. Marker represents a Pre-Stained Protein Ladder, Bid (overexpressed Bid), Vector (overexpressed vector), Mock (no transfection control), -ve siRNA (mission siRNA fluorescent universal negative control #1, Cyanine 3) and Bid siRNA (Mission esiRNA human BID).

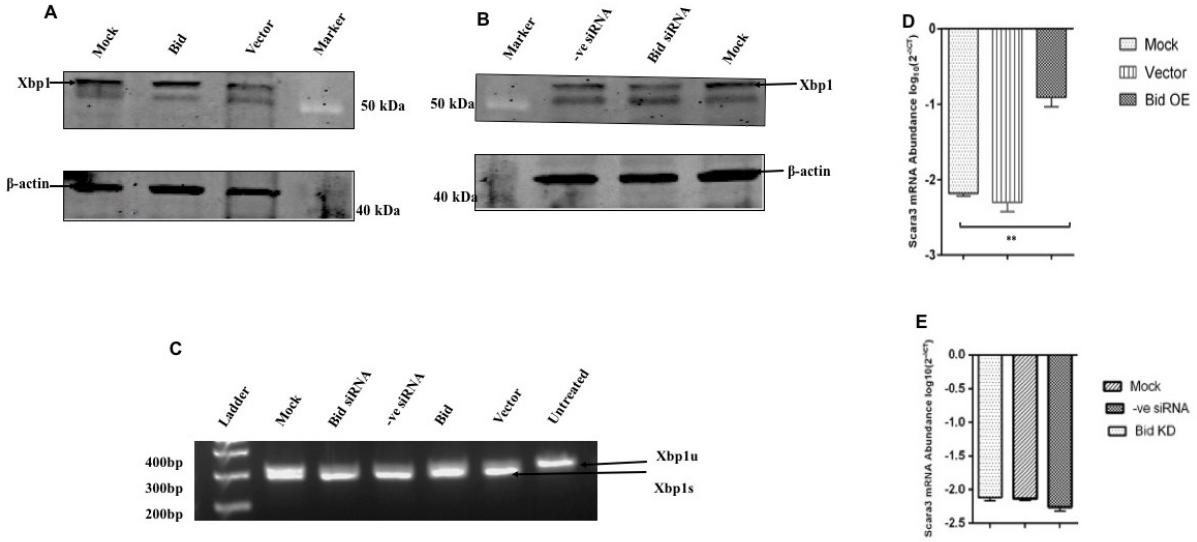

Figure3: Bid differentially modulates IRE1 RNAse outputs. A) HEK293-T cells were transiently transfected for Bid, Vector and Mock. After 42hr induction with 6μM tunicamycin was given for 6hr. Western blot was done to check the levels of spliced Xbp1 by probing with anti-Xbp1 antibody. B) Bid siRNA was transfected in HEK293-T cells along with a negative control siRNA. 6hr treatment with 6μM tunicamycin was given after 42hr of transfection. Western blot was done to check the levels of spliced Xbp1 by probing with anti-Xbp1 antibody. C) XBP-1 splicing detected by semi-quantitative RT-PCR. PCR products were separated on 2% agarose gel. The upper band defines unspliced Xbp1 (Xbp1u), where lower band is spliced Xbp1 (Xbp1s). D and E) Abundance of Scara3 mRNA was analysed by real time PCR. Real time PCR quantitation was done by plotting $\log_{10}$ of $2^{-\Delta CT}$ and represent mean SD values. D and E represent overexpression and knockdown respectively. Statistical analysis was performed using One way ANOVA: **: $p < 0.0016$.