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## The Immunity-related GTPase Irgm3 Relieves Endoplasmic Reticulum Stress Response during Coxsackievirus B3 Infection via a PI3K/Akt Dependent Pathway

Zhen Liu<sup>1</sup>, Huifang M. Zhang<sup>1</sup>, Ji Yuan<sup>1</sup>, Xin Ye<sup>1</sup>, Gregory A. Taylor<sup>2,3</sup>, and Decheng Yang<sup>1,\*</sup>

<sup>1</sup>Department of Pathology and Laboratory Medicine, University of British Columbia- The Heart + Lung Institute - St. Paul's Hospital, Vancouver, Canada

<sup>2</sup>Geriatric Research, Education, and Clinical Center, VA Medical Center, Durham, North Carolina

<sup>3</sup>Departments of Medicine, Molecular Genetics and Microbiology, and Immunology, Division of Geriatrics, and Center for the Study of Aging and Human Development, Duke University Medical Center, Durham, North Carolina, USA

### Abstract

The IRG protein Irgm3 preserves cell survival during coxsackievirus B3 (CVB3) infection. However, the molecular mechanisms are not clear. Here, we examined the effect of Irgm3 expression on ER stress triggered by pharmacological agents or CVB3 infection. In Tet-On/Irgm3 HeLa cells, Irgm3 expression suppressed either chemical- or CVB3-induced upregulation of glucose-regulated protein78. Further, Irgm3 strongly inhibited the activation of both the PERK and ATF6 pathways of ER stress responses, which further led to the diminished phosphorylation of eIF2 $\alpha$ , reduced cleavage/activation of transcription factor SREBP1 and attenuated induction of proapoptotic genes CHOP and GADD34. These data were further supported by experiments using Irgm3 knockout mouse embryonic fibroblasts, in which the ER stress induced by CVB3 was not relieved due to the lack of Irgm3 expression. In addition, the tunicamycin-triggered ER stress promoted the subsequent CVB3 infection. The effect of Irgm3 on ER stress and CVB3 infection was diminished by the PI3K inhibitor, LY294002, while inhibitors of ERK, JNK and p38 had no effect. These data were further corroborated by transfection of cells with a dominant negative Akt. Taken together, these data suggest that Irgm3 relieves the ER stress response via a PI3K/Akt dependent mechanism, which contributes to host defense against CVB3 infection.

### Introduction

Endoplasmic reticulum (ER) is the production site for cell membranes, secretory proteins, lipids, and sterol. The synthesized proteins are folded and glycosylated in the ER lumen with the aid of molecular chaperons before their budding and transport to Golgi apparatus. A variety of cellular stress conditions that interfere with ER function can cause the accumulation of misfolded/unfolded proteins in ER, and thus initiates a group of signal transduction pathways known collectively as the ER stress response or unfolded protein response (UPR), which is an effort of the cells to restore ER homeostasis (Lin *et al.*, 2008a, Yoshida, 2007, Szegezdi *et al.*, 2006). Three ER transmembrane proteins, the protein kinase-like ER resident kinase (PERK), the activating transcription factor 6 (ATF6) and the inositol-requiring enzyme 1 (IRE1), are mediators of the ER stress response. In normal conditions, these ER stress sensors are maintained in an inactive state through association

\*Correspondence to: Dr. Decheng Yang, The Heart + Lung Institute, University of British Columbia, St. Paul's Hospital, 1081 Burrard Street, Vancouver, B.C. Canada V6Z 1Y6. Phone: (604)682-2344 ext 62872; Fax: (604)806-9274; Decheng.yang@hli.ubc.ca.

with the glucose-regulated protein 78 (GRP78, also known as BiP), the most abundant chaperone in ER. Under stress conditions when GRP78 is sequestered to misfolded proteins, the PERK, ATF6, and IRE1 are released and activated sequentially through either autophosphorylation or translocation. Activation of PERK leads to attenuation of cellular protein synthesis via phosphorylation of eIF2 $\alpha$ ; activated ATF6 stimulates the transcription of chaperone genes; activation of IRE1 leads to the induction of protein degradation enzymes. However, if such concerted cellular responses fail to restore normal ER function, they also initiate apoptosis through enhancing expression of proapoptotic genes such as the proapoptotic transcription factor C/EBP homologous protein (CHOP), the growth-arrest- and DNA-damage-inducible gene 34 (GADD34) (Szegezdi *et al.*, 2006, Yoshida, 2007, Boyce *et al.*, 2006, Schroder, 2008).

With functions essential to cell survival, ER is also a host organelle utilized by many viruses for replication and maturation during infections (He, 2006, Braakman *et al.*, 2000). Viruses can induce ER stress either through overwhelming the protein folding capability, or directly disrupting ER structure and function, and in many cases, the virus-triggered ER stress responses were shown to facilitate virus replication and pathogenesis (Huang *et al.*, 2005, Smith *et al.*, 2006, Yu *et al.*, 2006, Li *et al.*, 2007). Coxsackievirus B3 (CVB3), a member of the picornavirus family, has been recognized as the most common infectious cause of myocarditis (Knowlton, 2008, Tam, 2006). It has been shown that picornaviruses require ER membranes for viral protein biogenesis and anchoring the replication complexes (Bienz *et al.*, 1990, Egger *et al.*, 2005, Suhy *et al.*, 2000). Certain coxsackievirus nonstructural proteins, such as 2B, 2C, and 3A, have been implicated in disrupting ER integrity and functions (van Kuppeveld *et al.*, 1997, Doedens *et al.*, 1997, Suhy *et al.*, 2000). Recently, our group has demonstrated that CVB3 infection induces ER stress response in both HeLa cells and cardiomyocytes (Zhang *et al.*, 2010).

Irgm3, formerly known as interferon- $\gamma$ -inducible GTPase, is a member of the GMS (or IRGM) subfamily in the family of 47-kDa IFN- $\gamma$ -responsive GTPases (immunity-related GTPases family, IRG). Three members (Irgm1–3) of this subfamily all contain a sequence GX4GMS in the first nucleotide-binding motif (Bekpen *et al.*, 2005). Accumulated data has shown that proteins in this IRG family play critical roles in mediating interferon- $\gamma$ -induced resistance to specific intracellular pathogens, which include protozoa, bacteria and viruses (Taylor *et al.*, 2000, Singh *et al.*, 2006, Taylor *et al.*, 2004). Irgm3 localizes predominantly to ER, and has been found to be essential for host resistance to acute infection by the protozoans *Toxoplasma gondii* and *Leishmania major* (Halonen *et al.*, 2001, Taylor *et al.*, 2004). Our previous study also demonstrated that Irgm3 is significantly up-regulated in CVB3-infected mouse hearts (Yang *et al.*, 1999), and Irgm3 expression inhibits CVB3-induced apoptosis through activation of the prosurvival pathway and inhibition of viral replication (Zhang *et al.*, 2003, Liu *et al.*, 2008). However, the molecular mechanisms of its prosurvival and anti-virus function are still poorly understood.

In this study, using both the doxycycline (Dox)-inducible Tet-On/Irgm3 HeLa cells overexpressing Irgm3 and the Irgm3 knockout (KO) mouse embryonic fibroblast (MEF) cells, we showed that Irgm3 expression relieves ER stress responses that are induced either by chemical agents or by CVB3 infection. We demonstrated that Irgm3 expression inhibited CVB3-induced upregulation of GRP78. We further showed that Irgm3 inhibited the activation of both PERK and ATF6 pathways of UPR, and suppressed the downstream apoptotic gene expression. Compared to the Tet-On/Irgm3 cells and wild type (WT) MEF, the alleviation of ER stress response was not observed in Irgm3 KO MEF cells under interferon- $\gamma$  treatment, which further suggest that Irgm3 is the molecule responsible for relieving ER stress response. Moreover, by experiments using specific inhibitors and dominant negative plasmids, we found that the relief of ER stress response by Irgm3

expression is dependent on the activation of the PI3K/Akt pathway, but not the MAPK pathways.

## Results

### Irgm3 expression relieves chemical-induced ER stress response

Previously using a Dox-inducible Tet-On/Irgm3 HeLa cell line, we have demonstrated that Irgm3 expression can promote cell survival through activating FAK-PI3K/Akt signaling pathway (Zhang *et al.*, 2003, Liu *et al.*, 2008). In the present study, we further investigated the influence of Irgm3 expression on ER stress response using this established Irgm3-inducible cell line. We first confirmed, by Western blotting, that Dox itself and the subsequent overproduction of Irgm3 have no effect on GRP78 expression in normal condition (Fig. 1A). We then investigated whether Irgm3 expression modulates the ER stress response caused by classical ER stress inducers tunicamycin (Tu) and brefelding A (BFA). Tu is an ER stress inducer that functions by inhibiting protein N-linked glycosylation, while BFA causes the accumulation of unfolded proteins by blocking ER to Golgi trafficking. GRP78 is a major chaperone and stress-sensing protein in ER, and its upregulated expression is recognized as the marker of the ER stress response (Schroder, 2008, Yoshida, 2007). As shown in Fig. 1B, tunicamycin treatment of the Tet-On/Irgm3 HeLa cells led to the activation of UPR, i.e. the gradual increase of GRP78 expression, in a dose-dependent manner. The substantial upregulation of GRP78 occurred only after 12 h of Tu exposure (Supplement Fig. S1). However, when Irgm3 was induced with Dox, the upregulation of GRP78 was significantly inhibited, particularly in the cells treated with a dose of 1  $\mu$ g/ml Tu, as compared to the corresponding uninduced cells. The changes of GRP78 mRNA levels were also detected and the data is in accordance with the protein expression (Fig. S2). The inhibitory effect of Irgm3 on UPR was further verified by experiments using another pharmacological agent BFA, in which the dose-dependent upregulation of GRP78 by BFA treatment was significantly inhibited by the Dox-induced Irgm3 expression (Fig. 1C). Thus, Irgm3 relieves the ER stress response triggered by different ER stress inducers.

To explore if the role of GTPase function (the role of GMS motif) of Irgm3 is important in relieving ER stress response, a pTRE-Irgm3/GMN plasmid inducibly expressing a mutant Irgm3 was used to transfect premade Tet-On HeLa cells and then test if it can affect the relieving of Tu-induced ER stress response after Dox induction. Fig. 1D demonstrated that Wt and GMN mutant of Irgm3 (S98N) have the same impact on GRP78 expression; thus, Irgm3-mediated relief of ER stress response seems independent of the GTPase function.

### Irgm3 expression inhibits the activation of both PERK and ATF6 pathways of ER stress response

ER stress responses consist of three branches of response pathway, PERK, ATF6, and IRE1. Among them, the PERK and ATF6 pathways are believed to be activated in the early phase of stress conditions, playing determinant roles in the outcome of ER stress (Schroder, 2008, Boyce *et al.*, 2006). To explore how Irgm3 expression affects ER stress response, activation of PERK and ATF6 pathways was investigated respectively. Activation of PERK pathway was examined by detection of phosphorylation of both PERK (p-PERK) and its only target, the translational initiation factor eIF2 $\alpha$  (p-eIF2 $\alpha$ ) by Western blot. As shown in Fig. 2, PERK phosphorylation was significantly increased at 16 h post Tu treatment at the concentration of 1  $\mu$ g/ml, and accordingly, eIF2 $\alpha$  was also activated through phosphorylation without changes on its total protein levels. However, the p-PERK and p-eIF2 $\alpha$  were markedly decreased almost to the level of the untreated control following the induction of Irgm3 expression with Dox (Fig. 2). Although causing translational attenuation,

phosphorylation of eIF2 $\alpha$  also paradoxically enhances the expression of activating transcription factor 4 (ATF4), which can stimulate the transcription of apoptotic genes in ER stress (Yoshida, 2007). Thus, the expression of ATF4, the downstream gene of the PERK-eIF2 $\alpha$  cascade, was further determined. It was found that the upregulation of ATF4 levels after Tu treatment was strongly inhibited by Irgm3 expression in parallel with the changes of p-PERK and p-eIF2 $\alpha$  (Fig. 2), indicating the overall inhibition of the PERK pathway by Irgm3 expression.

After dissociation from GRP78 under ER stress, the ATF6 proform (90 kDa) translocates to the Golgi where it is cleaved into its active form (50 kDa) by proteinases. Active ATF6 then moves to the nucleus and induces transcription of ER chaperon genes (Yoshida, 2007, Schroder, 2008). To assess the activation of ATF6, the ATF6 proform and the cleavage products were probed in the cytosolic and the nuclear fractions respectively by Western blot. Here, histone-1 was used as a nuclear protein loading control in addition to the cytoplasmic  $\beta$ -actin control to further validate the fractionation technique (Fig. S3). We showed that Tu treatment alone caused a very evident loss of ATF6 proform in the cytosol, and an elevated level of the active form in the nucleus; whereas with the Irgm3 expression, the ATF6 in the cytosol was largely reserved as compared with the uninduced and Tu-treated cells (Fig. 3). Here it is worth to point out that the endogenous cleaved ATF6 in lane 1 of Fig 3 is relatively high, which is likely due to the use of the Tet-On HeLa cell line. The notion that the basal level of the cleaved ATF6 in Tet-On cells can be higher than that in the regular HeLa cells is supported by the different endogenous levels of GRP78 and GADD34 among the tested cell lines (Fig. S4).

### **Irgm3 attenuates the ER stress-mediated apoptotic response**

As aforementioned, if the initial stress responses cannot resolve the accumulation of misfolded/unfolded proteins and restore normal ER function promptly, the signal pathway will eventually shift from ER stress response to programmed cell death. To determine whether Irgm3 expression can block or attenuate such transition, crucial mediators (CHOP, GADD34, sterol regulatory element binding protein-1 (SREBP1) and caspase-3) involved in the apoptosis mediated by ER stress response were examined by Western blot analysis. As shown in Fig. 4, the proapoptotic transcription factor CHOP was strongly upregulated after Tu treatment. Further, the GADD34 expression also showed upregulation in these Tu treated cells. It is known that CHOP upregulation subsequently leads to increased production of downstream GADD34 (Marciniak *et al.*, 2004), and our data support this previous report. Fig. 4 further shows that SREBP1, another proapoptotic transcription factor involved in lipid metabolism in ER (Wang *et al.*, 2005, Zhou *et al.*, 2006, Marciniak *et al.*, 2006), was also activated by cleavage upon Tu treatment. However, the upregulation of these apoptotic mediators was significantly suppressed when the Irgm3 expression was induced with Dox. These data were further solidified by examining the downstream apoptotic events including both the activation of apoptosis executioner caspase-3 and the cleavage of its substrate, poly-(ADP-ribose) polymerase (PARP) (Szegezdi *et al.*, 2006, Duriez *et al.*, 1997). We showed that at 20 h post Tu treatment, the 19 kDa active form of executioner caspase-3 was dramatically increased and meanwhile so was the 89 kDa cleavage product of PARP in the Tu-treated cells. Nevertheless, these increases were significantly attenuated following the induction of Irgm3 expression with Dox (Fig. 4). These results suggest that Irgm3 expression relieves the ER stress-mediated apoptotic response.

### **Irgm3 relieves the ER stress response triggered by CVB3 infection and inhibits viral replication**

It has been found that CVB3 can trigger ER stress response, and eventually lead to cell apoptosis (Zhang *et al.*, 2010). To determine the effect of Irgm3 expression on CVB3-

induced ER stress responses, Tet-On/Irgm3 HeLa cells were infected with CVB3 at 10 multiplicity of infection (MOI) or sham-infected with PBS, and the expression of GRP78 as well as other signal molecules in the activated UPR pathways were then detected by Western blot. Compared to the sham-infected control, GRP78 expression in the uninduced/CVB3-infected cells gradually increased starting from 6 h post infection (pi) (Fig. 5A). However, for the infected cells that were pre-induced with Dox, such upregulation of GRP78 was largely inhibited, suggesting that Irgm3 can relieve CVB3-induced ER stress. To rule out the possibility that Dox or vector itself can attenuate the ER stress response, premade Tet-On HeLa cells (Clontech) containing the vector only were used to repeat the experiment as described above and it demonstrated that GRP78 expression in the Dox-treated and CVB3-infected premade Tet-On cells was upregulated in a similar dynamic and scaled manner (Fig. 5B) to that of the non-induced Tet-On IGTP cells (Fig. 5A), suggesting that Dox or the vector itself has no effect on GRP78 upregulation.

To determine whether this ER stress relief affects individual branch of UPR, the activation of the PERK and ATF6 pathways in the CVB3-infected Tet-On/Irgm3 cells was examined. Firstly, on the PERK pathway, CVB3 infection was found to cause a significant increase in both p-PERK and p-eIF2 $\alpha$  levels at 12 h pi; but in the Dox-induced cells, such upregulation was hardly detectable (Fig. 6A), indicating that Irgm3 expression inhibits the CVB3-induced activation of PERK pathway. Secondly, to examine the ATF6 pathway, the proform of ATF6 in the cytosolic fraction and the cleaved form in the nuclear fraction of CVB3-infected and sham-infected control cells were detected respectively. As shown in Fig. 6B, CVB3 infection at 10 MOI for 12 h caused a decrease of ATF6 proform in cytosol and a significant increase of its active form in the nucleus. However, after the induction of Irgm3 expression with Dox, the translocation of the active ATF6 into nucleus was largely diminished as compared to the uninduced controls. These results suggest that Irgm3 relieves CVB3-induced ER stress response by inhibiting the activation of both PERK and ATF6 pathways.

To further illuminate the significance of the ER-stress relief effect of Irgm3 on CVB3 infection, CVB3 replication efficiency in Tet-On/Irgm3 cells (induced or uninduced with Dox; treated or untreated with Tu) was determined. Firstly, different extent of preemptive ER stress response was induced by 1  $\mu$ g/ml Tu treatment for different periods after Dox induction and prior to CVB3 infection. Cells and supernatants were collected for Western blot analysis to measure CVB3 structural protein VP1, an indicator of viral protein synthesis, and for plaque assay to quantitate the infectious viral particles. As shown in Fig. 7A, viral VP1 protein synthesis in cells uninduced with Dox but treated with Tu was greatly increased, and the degree of such VP1 increase correlated well with the extent of ER stress response as indicated by the level of GRP78 expression. Also notably, the attenuation of GRP78 synthesis by Dox induction of Irgm3 was coupled with the reduction of VP1 production in each corresponding group. The plaque assay further showed that the ER stress response induced by Tu treatment for 12 h (Fig. 7B) or BFA treatment for 16 h (Fig. S5) markedly promoted viral release from CVB3-infected cells and this promotion was significantly reduced by Dox-induced Irgm3 expression. In addition, the total viral particles were determined by plaque assays using supernatants and cells after three freeze/thaw cycles (Fig. 7C). The correlation with the data in Fig. 7B indicates that ER stress response appears to be beneficial to CVB3 infection, and relieving ER stress response by Dox-induced Irgm3 inhibits CVB3 replication and also decreases viral particle release.

### **Irgm3 is responsible for IFN- $\gamma$ -induced relief of ER stress response during CVB3 infection**

Next, we addressed whether Irgm3 played a similar role in relieving ER stress response and in inhibiting CVB3 replication in MEF. As shown in Fig. 8A, WT MEF strongly expressed Irgm3 after IFN- $\gamma$  induction, while Irgm3 KO MEF lacked the expression, as expected.



When WT MEF cells were infected with CVB3 at an MOI as high as 40, the viral VP1 protein was detected 12 h pi. However, when these cells were pretreated with IFN- $\gamma$  prior to infection, VP1 protein synthesis was dramatically reduced (Fig. 8B). In comparison, the effect of IFN- $\gamma$  on VP1 synthesis (97% reduction) was markedly attenuated in Irgm3 KO MEF (69% reduction). Consistently, the viral plaque assays of the supernatants (Fig. 8C) as well as the supernatants plus the cell lysates (Fig. 8D) showed that the inhibition of CVB3 replication and particle release by IFN- $\gamma$  was also markedly attenuated in Irgm3 KO MEF, which again supports that Irgm3 plays a major role in the IFN- $\gamma$ -induced antiviral effect. We next tested whether Irgm3 has an effect on the previously reported modulation of the ER stress response by IFN- $\gamma$  (Lin *et al.*, 2008b, Lin *et al.*, 2007). Transient IFN- $\gamma$  treatment in our experiments significantly diminished the upregulation of GRP78 and ATF4 and the activation of PERK signals upon CVB3 infection in WT MEF (Fig. 8B). However, notably, such attenuation of GRP78 and ATF4 expression as well as phosphorylation of PERK and eIF2 $\alpha$  by IFN- $\gamma$  treatment all appeared to be largely decreased or lost in the Irgm3 KO cells, suggesting that Irgm3 is one of the major IFN- $\gamma$ -inducible genes responsible for relieving the CVB3-induced ER stress response in MEF.

### The relief of ER stress response by Irgm3 depends on PI3K/Akt activation

Various cell signaling pathways, particularly the PI3K/Akt and MAPK pathways, have been implicated in the activation or feedback of the ER stress response (Urano *et al.*, 2000, Su *et al.*, 2002, Kim *et al.*, 2009, Hu *et al.*, 2004). It is known that Irgm3 activates the PI3K/Akt pathway (Zhang *et al.*, 2003). Therefore, we assessed whether this or other pathways are involved in the effect of Irgm3 on ER stress response. Specific inhibitors of JNK, ERK, p38 MAPK and PI3K/Akt pathways were applied to Tet-On/Irgm3 HeLa cells prior to Tu treatment. Only the PI3K/Akt inhibitor, LY294002, showed a significant effect on GRP78 and p-Akt levels, i.e., the Irgm3-mediated suppression of GRP78 induction was largely reversed and the Tu-mediated inhibition of phosphorylation of Akt was greatly enhanced (Fig. 9A). The same inhibitors were also used to examine the Irgm3-mediated rescue of Tu-treated cells (Fig. 9B). Again, only the PI3K/Akt inhibitor, LY294002, significantly decreased the cell viability, almost eliminating the protective effect of Irgm3 during ER stress (Fig. 9B).

To further solidify the data on the dependence of Irgm3 on the PI3K/Akt pathway in ER stress relief, PI3K/Akt activity was inhibited through expression of dominant negative (DN)-Akt or DN-FAK (focal adhesion kinase). FAK is positioned upstream of Akt (Liu *et al.*, 2008); thus, DN-FAK, also called FRNK (FAK-related non-kinase), would have an analogous effect on knocking down the Akt activity. Similar to the results of the inhibitor experiment, the relief effect of Irgm3 on ER stress response appeared to be blocked by the inhibition of phosphorylation of Akt. As shown in Fig. 9C, the Tu-induced upregulation of GRP78 was restored by transfection of the cells with either DN-Akt or FRNK as compared to the vector-transfected control cells. Furthermore, the enhanced cell survival by Irgm3 expression was also abrogated specifically by DN-Akt or FRNK as compared to the controls (Fig. 9D). Taken together, these results suggest that the relief of ER stress response by Irgm3 expression is dependent on the activation of PI3K/Akt pathway.

## Discussion

Irgm3, an IFN- $\gamma$  inducible GTP-binding protein localized in ER, has been previously shown by our laboratory to mediate cell resistance to CVB3 infection and preserve cell survival through activating the FAK-PI3K/Akt signaling pathway (Zhang *et al.*, 2003, Liu *et al.*, 2008). In spite of these findings, the prosurvival mechanisms of Irgm3 during viral infection as well as the implication of the ER localization of Irgm3 still remains largely unclear. In the present study using cells either inducibly overexpressing Irgm3 or lacking of Irgm3

expression, we found that *Irgm3* relieves the ER stress response that is triggered either by pharmacological agents or by CVB3 infection. In experiments using two types of ER stress inducers, i.e., Tu and BFA, inhibitors of protein glycosylation and of vesicle trafficking, respectively, we showed that *Irgm3* alleviated the dose-dependent ER stress response triggered by either chemical agent. Moreover, the intensity, duration or periodicity of ER stress experienced by cells during viral infection could be quite different from those caused by chemical inducers (Boyce *et al.*, 2006). For example, CVB3-induced ER stress response occurred earlier (6 h pi) than those induced by Tu or BFA (12–20 h post treatment). However, the relief effect of *Irgm3* on ER stress response was fully recapitulated in CVB3 infection, and *Irgm3* expression could significantly relieve both types of ER stress responses. The *Irgm3*-mediated relief of ER stress response in CVB3 infection was further verified by experiments using WT and *Irgm3* KO MEF, in which GRP78 levels in IFN- $\gamma$ -treated WT MEF during infection were significantly lower than those in the *Irgm3* KO cells. Taken together, these results demonstrate that *Irgm3* expression relieves the ER stress response triggered by various inducers including CVB3.

To further specify the effect of *Irgm3* on individual branches of ER stress response, we examined the activation of PERK and ATF6 pathways that react usually at the early stage of stress (Szegezdi *et al.*, 2006, Harding *et al.*, 1999). We found that *Irgm3* inhibited the activation of both PERK and ATF6 pathways during ER stress. The phosphorylation of PERK has been known as an earliest event during ER stress (Harding *et al.*, 1999). Phosphorylation of eIF2 $\alpha$  by activated PERK at Ser51 inhibits cap-dependent translation of the majority of cellular mRNAs. However, ATF4 can escape this translational attenuation by multiple upstream open reading frames and the increased ATF4 further stimulates the expression of CHOP (Averous *et al.*, 2004). Our data showed that *Irgm3* expression inhibited the activation of this entire PERK signaling cascade including the phosphorylation of PERK and eIF2 $\alpha$ , and the upregulation of ATF4 expression. It is known that ATF6 pathway responds to ER stress by the cleavage and then translocation of the active ATF6 to the nucleus, where it upregulates the transcription of target genes that overlap much with those activated by ATF4 (Szegezdi *et al.*, 2006, Schroder, 2008). Herein, *Irgm3* was found to also block the activation of ATF6 pathway as demonstrated by reducing nuclear translocation of the active ATF6 fragment. Therefore, the effect of *Irgm3* on ER stress responses appears to be global, rather than pathway-exclusive.

Prolonged ER stress is considered to be one of the major triggers of apoptosis and can activate a combination of intrinsic and extrinsic apoptotic pathways (Tajiri *et al.*, 2004, Nakagawa *et al.*, 2000, Rasheva *et al.*, 2009). Although it may be transiently favorable for cell survival under mild stress, activation of PERK and ATF6 eventually serves to shift the pro-survival response to pro-apoptotic signaling (Szegezdi *et al.*, 2006, Boyce *et al.*, 2006). Particularly, the activation of PERK leads to the induction of pro-apoptotic CHOP via ATF4, which is the key element in ER stress-mediated apoptosis (Szegezdi *et al.*, 2006). Accordingly, the inducers in our study triggered the eventual shift from ER stress to cell apoptosis and this transition appears to occur through activation of the well recognized ATF4-CHOP-GADD34 cascade (He, 2006, Marciniak *et al.*, 2004), which initiated the activation of executioner caspase-3 and subsequent cleavage of PARP. Nevertheless, these proapoptotic signals and subsequent apoptotic events were markedly suppressed by ectopic *Irgm3* expression. Correspondingly, cell viability was also greatly enhanced thereafter. These data are also consistent with the previous observation that *Irgm3* preserves the cell viability during viral infection (Zhang *et al.*, 2003). Thus, these results suggest that *Irgm3* blocks the initiation of the pro-apoptotic pathway and preserves cell survival during ER stress through inhibition of PERK and ATF6 pathways.

An increasing number of viruses have been reported to be able to manipulate the ER stress response for their own advantages (Alwine, 2008, Huang *et al.*, 2005, Tardif *et al.*, 2004). Both the PERK and ATF6 pathways have been shown to be activated in various viral infections (Baltzis *et al.*, 2004, Netherton *et al.*, 2004, Jordan *et al.*, 2002, Cheng *et al.*, 2005), and in many cases, the activation of these two pathways were shown to facilitate virus propagation (Smith *et al.*, 2006, Tardif *et al.*, 2002). CVB3 is a picornavirus capable of synthesizing its proteins by a cap-independent mechanism of translation initiation (Trono *et al.*, 1988). Thus, the eIF2 $\alpha$  phosphorylation by activated PERK in ER stress response would inhibit the cap-dependent translation of cellular proteins, providing more translational machinery available for CVB3, and may thereby enhance the viral protein synthesis (Zhang *et al.*, 2010, Yang *et al.*, 1997). For this reason, in the present study, with the relief of CVB3-triggered ER stress response by Irgm3 expression, viral VP1 protein synthesis was shown to be reduced. Moreover, the preemptive ER stress response appears to aid both the protein synthesis and replication of CVB3, which further underscores the significance of ER stress relief in controlling CVB3 infection. This connection was further supported by experiments using Irgm3 KO cells, in which the abrogation of the Irgm3-mediated relief of ER stress was accompanied by the attenuated inhibition of CVB3 protein production under IFN- $\gamma$  treatment. These results suggest that Irgm3, one of the many IFN- $\gamma$ -inducible genes, plays a key role in relieving ER stress response, with such an effect being an important anti-CVB3 mechanism of Irgm3.

The association of various signaling transduction pathways with the ER stress response has been increasingly substantiated, and recent reports have illuminated the connections between ER stress response and the PI3K/Akt, ERK, JNK, and p38 MAPK pathways in different setting of studies, comprising reciprocal regulation and negative feedback (Urano *et al.*, 2000, Kim *et al.*, 2009, Hu *et al.*, 2004, Du *et al.*, 2003). In the present study, we showed that among all the above tested signals, only inhibition of PI3K/Akt pathway with either specific inhibitor or dominant negative mutants could resuscitate the upregulation of GRP78 during ER stress and abrogate the Irgm3-enhanced cell viability, suggesting the dependence of Irgm3 on PI3K/Akt pathway for ER stress relief. Multiple interactions between PI3K/Akt survival pathways and ER stress responses have been identified previously, such as those through eIF2 $\alpha$ -CHOP-TRB3 or p58<sup>IPK</sup> signaling, where the Akt pathway was shown to be essential to protect cells from ER stress-induced apoptosis (Ohoka *et al.*, 2005, Du *et al.*, 2003, Hu *et al.*, 2004, Szegezdi *et al.*, 2006). In consistence with the previous finding that Akt pathway is pivotal for the prosurvival effect of Irgm3, our results suggest that the Irgm3-mediated relief of ER stress response depends on the activation of PI3K/Akt pathway, but not the MAPK pathways.

In summary, our study has demonstrated that Irgm3 expression relieves ER stress responses triggered by either chemical inducers or CVB3 infection. We also found that Irgm3 protected cell from ER stress-mediated apoptosis by inhibiting the activation of the PERK and ATF6 ER stress sensors. Our results further suggest that the Irgm3-mediated relief of ER stress depends on the activation of PI3K/Akt pathway. Moreover, the relief effect of Irgm3 on ER stress responses not only contributes to ER homeostasis but also helps restrict CVB3 infection, which further highlights that Irgm3 may serve as a potential candidate in developing anti-CVB3 therapeutics.

## Experimental procedures

### Cells and virus

CVB3 (CG strain) was routinely propagated in HeLa cells (ATCC). The virus suspension was obtained by three cycles of freeze-thaw and centrifugation to remove cell debris and stored at  $-80^{\circ}\text{C}$ . Virus titers were determined by plaque assay prior to infection. A



previously established double-stable Tet-On/Irgm3 HeLa cell line was used in this study (Liu *et al.*, 2008). Tet-On/Irgm3 HeLa cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM glutamine, 1 mM HEPES, and 10% Clontech-approved fetal bovine serum specially prepared for Tet-On system in the presence of both 100  $\mu$ g/ml G418 and 100  $\mu$ g/ml hygromycin. MEF cells were propagated in Eagle's medium supplemented with 10% fetal bovine and 2 mM L-glutamine.

### Antibodies, plasmids and reagents

Monoclonal antibodies against GRP78, p58<sup>IPK</sup> p-eIF2 $\alpha$ , PARP, Caspase 3, CHOP, total eIF2 $\alpha$ , Irgm3,  $\beta$ -actin, were purchased from Cell Signaling Technology. Monoclonal antibodies against p-PERK, ATF6, SREBP1, ATF4, GADD34, total PERK, and Histone 1 were purchased from Santa Cruz Biotechnology. The monoclonal anti-VP1 antibody was purchased from DakoCytomation. Goat antibodies against mouse IgG and rabbit polyclonal antibody were obtained from BD Biosciences and Santa Cruz Biotechnology, respectively. The ER stress inducers Tu and BFA were purchased from Sigma-Aldrich and eBioscience, respectively. Recombinant IFN- $\gamma$  was purchased from Cell Sciences. The PI3K inhibitor LY294002 was purchased from Cell Signaling. ERK inhibitor U0126 was purchased from Promega. JNK inhibitor SP600125 and p38 inhibitor SB203580 were purchased from Calbiochem. The dominant negative mutant Akt1 construct was from Upstate Biotechnology. The pTRE-Irgm3 plasmid and its mutant pTRE-Irgm3/S98N were constructed previously by Dr. Gregory A. Taylor (Taylor *et al.*, 1997). The pHK-FRNK construct was obtained from Dr. Junlin Guan, University of Michigan.

### Western blot analysis

Cells were washed twice with ice-cold PBS containing complete phosphatase inhibitor (Roche) and cell lysates were prepared as described previously (Zhang *et al.*, 2003). The protein concentration was determined by the Bradford assay (Bio-Rad). Twenty to 80 micrograms of extracted proteins were fractionated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blocked with PBS containing 0.1% Tween 20 and 5% nonfat dry milk for 40 min. Afterward, the membrane was incubated with the specific primary antibody overnight at 4°C, followed by the secondary antibody for 1 h at room temperature. The immunoblots were visualized with an enhanced chemiluminescence detection system according to the protocol of the manufacturer (Amersham Pharmacia Biotech). Densitometry analysis was performed by using the Genetools software (Syngene). Density values for proteins were normalized to the level of control groups (arbitrarily set to 1.0).

### Cell fractionation

NE-PER® nuclear and cytoplasmic extraction reagents from Pierce Biotechnology was used to separate nuclear and cytosol proteins. Cell lysates from each fraction were prepared following the manufacturer's instructions, and the protein contents were measured using the Bradford method.

### Cell viability assay

Cell viability following virus infection was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay reagents, according to the manufacturer's instructions (Promega). Briefly, cells were incubated with MTS solution for 2 h and absorbance was measured at 492 nm using an enzyme-linked immunosorbent assay plate reader (Tecan, spectra fluoro plus). The

absorbance of sham-infected cells was defined as a value of 100% survival and the remaining data were converted to the ratio of the sham-infected sample.

### Viral plaque assay

Cells either untreated or treated with Tu were infected with CVB3 at a MOI of 10 for 1 h. Cells were washed and replenished with serum-free DMEM for 9 h. The supernatants were collected to determine viral titer on HeLa cell monolayers in triplicate following the standard procedures described previously (Zhang *et al.*, 2003). Agar overlays were fixed and cells were stained with crystal violet solution to visualize plaques at day 3 after infection. The virus titer was calculated as plaque forming unit (pfu) per milliliter.

### Statistical analysis

Two-way analysis of variance with multiple comparisons and paired Student's *t* test was performed. Values shown are the mean  $\pm$  standard error. A *P* value of  $<0.05$  was considered statistically significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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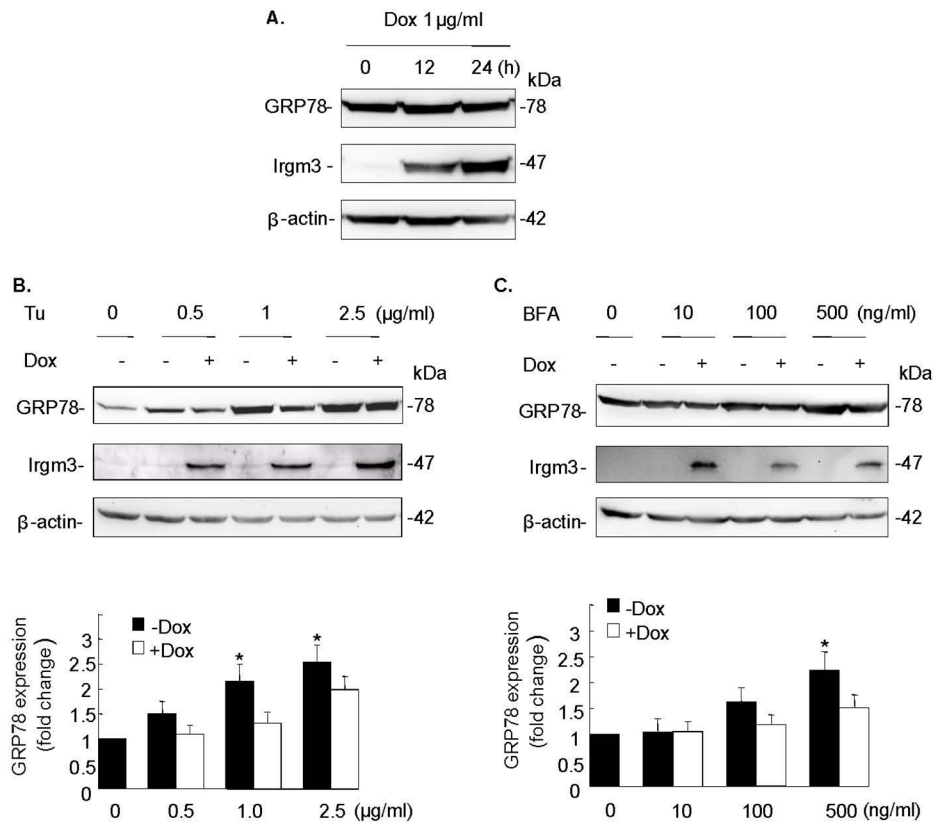
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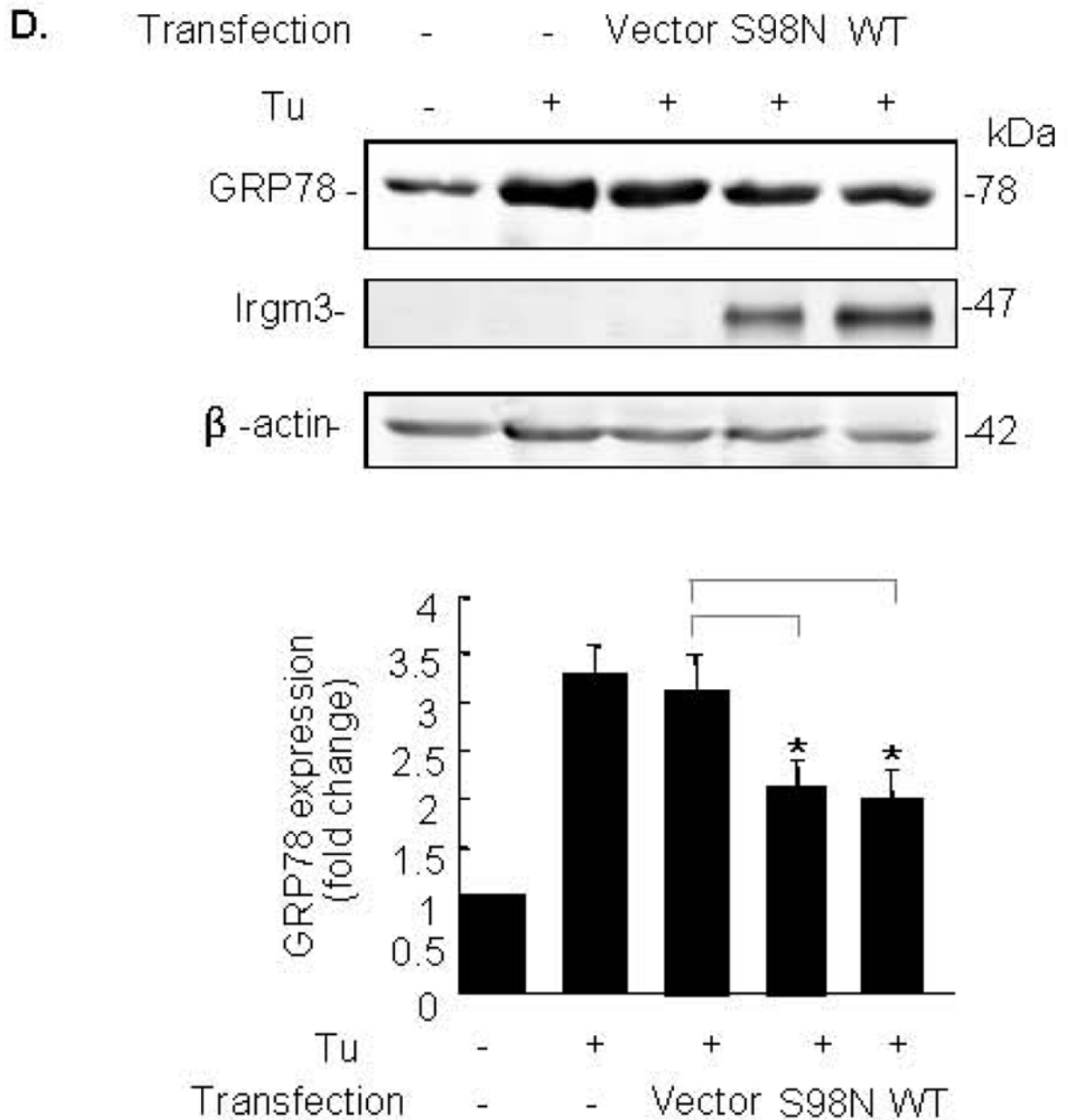
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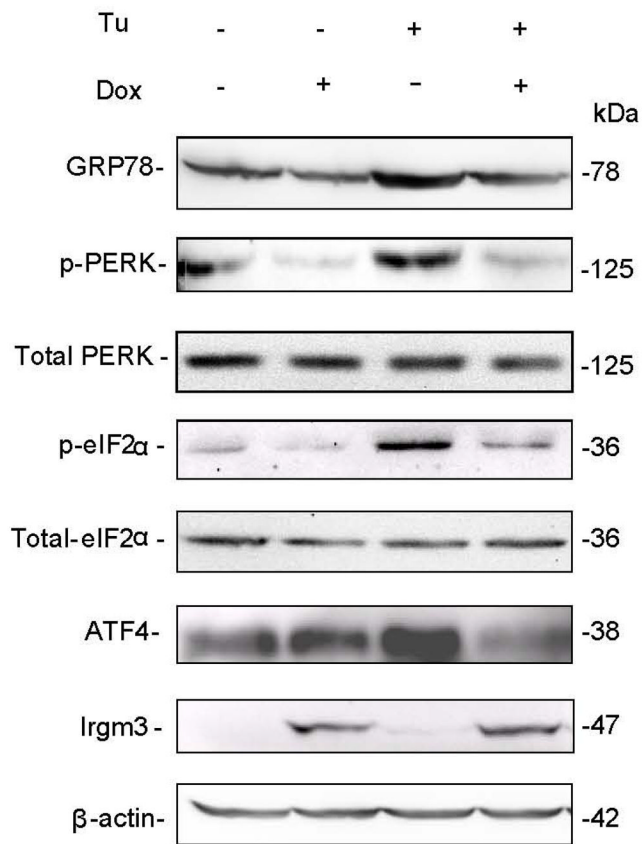




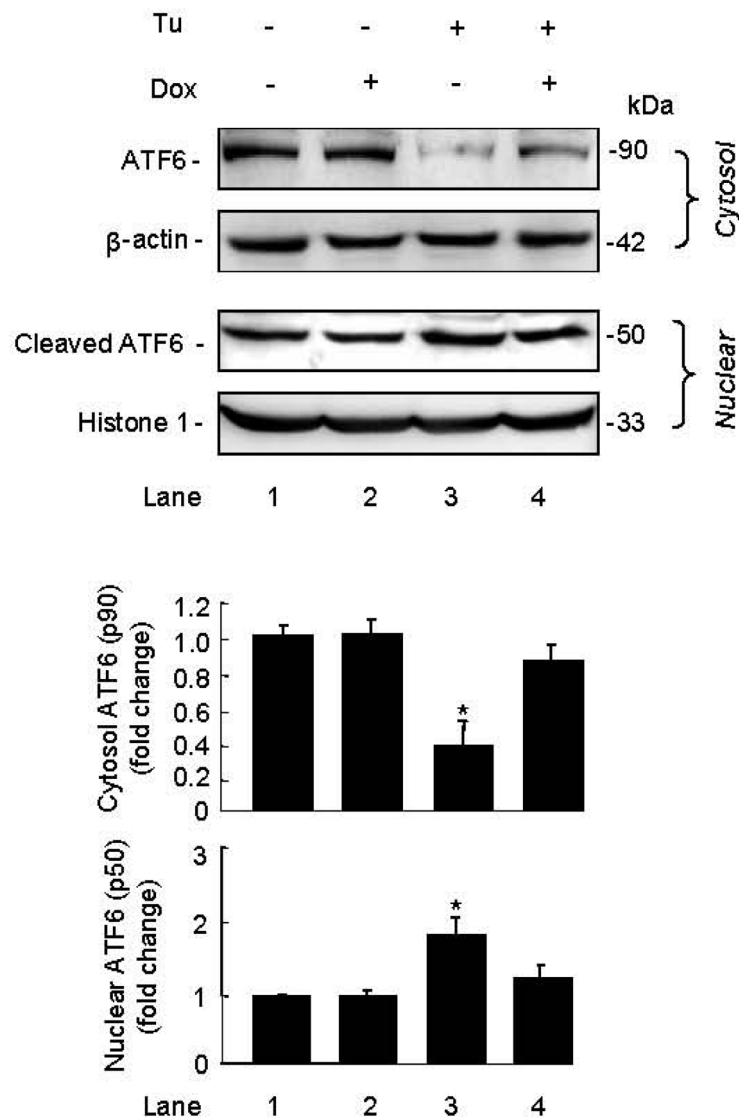
**Figure 1. Irgm3 relieves ER stress response triggered by chemical inducers**

Tet-On/Irgm3 HeLa cells were induced with 1  $\mu$ g/ml Dox, and Western blot analysis was conducted to confirm that Dox itself has no effect on GRP78 expression in normal condition (A). Tet-On/Irgm3 HeLa cells were induced with 1  $\mu$ g/ml Dox for 16 h and then treated with Tu (B) or BFA (C) at the indicated concentrations. Cell lysates were harvested at 16 h and 20 h post treatment with Tu and BFA respectively, and then subjected to immunoblotting using indicated antibodies. (D). Premade Tet-On HeLa cells were transfected with pTRE vector, pTRE-Irgm3/S98N mutant or pTRE-WT Irgm3, and induced with Dox for 36 hrs. Then, cells were treated with 1  $\mu$ g/ml Tunicamycin and cell lysates were collected for immunoblotting. All Data were quantitated by densitometric analysis and

normalized to the  $\beta$ -actin expression. Untreated controls were arbitrarily set to 1.0, and expression of GRP78 was calculated as fold increase as compared to the control group. Data represents the mean  $\pm$  S.E. from three independent experiments (\* $p < 0.05$ ).



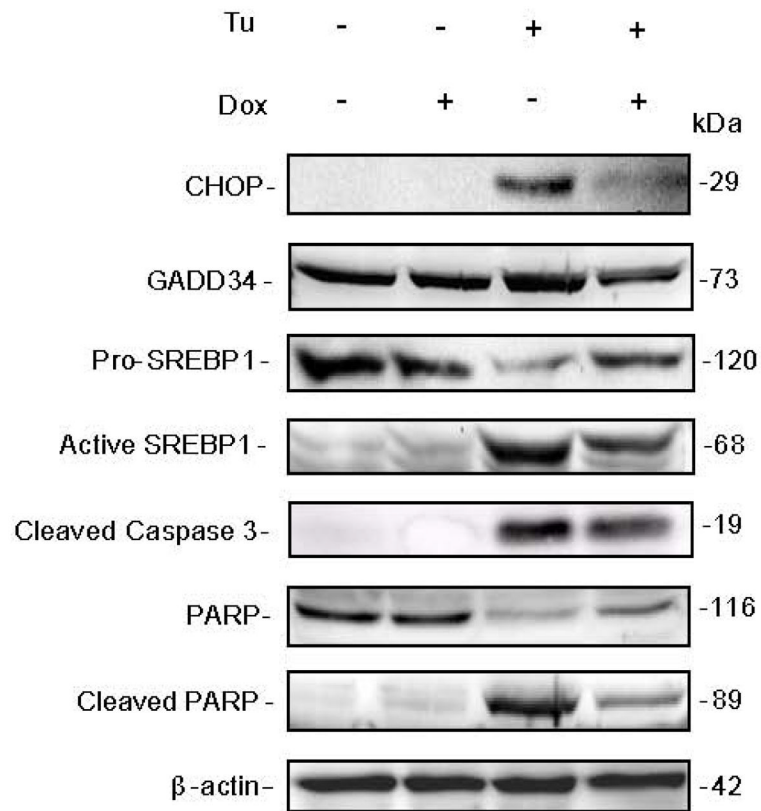
**Figure 2. Irgm3 inhibits the activation of PERK pathway of ER stress response**  
 Tet-On/Irgm3 HeLa cells were induced with 1  $\mu$ g/ml Dox for 16 h and then treated with 1  $\mu$ g/ml Tu for 16 h or untreated. Cell lysates were subjected to immunoblot analysis with indicated antibodies. Detection of  $\beta$ -actin was used as the loading control.



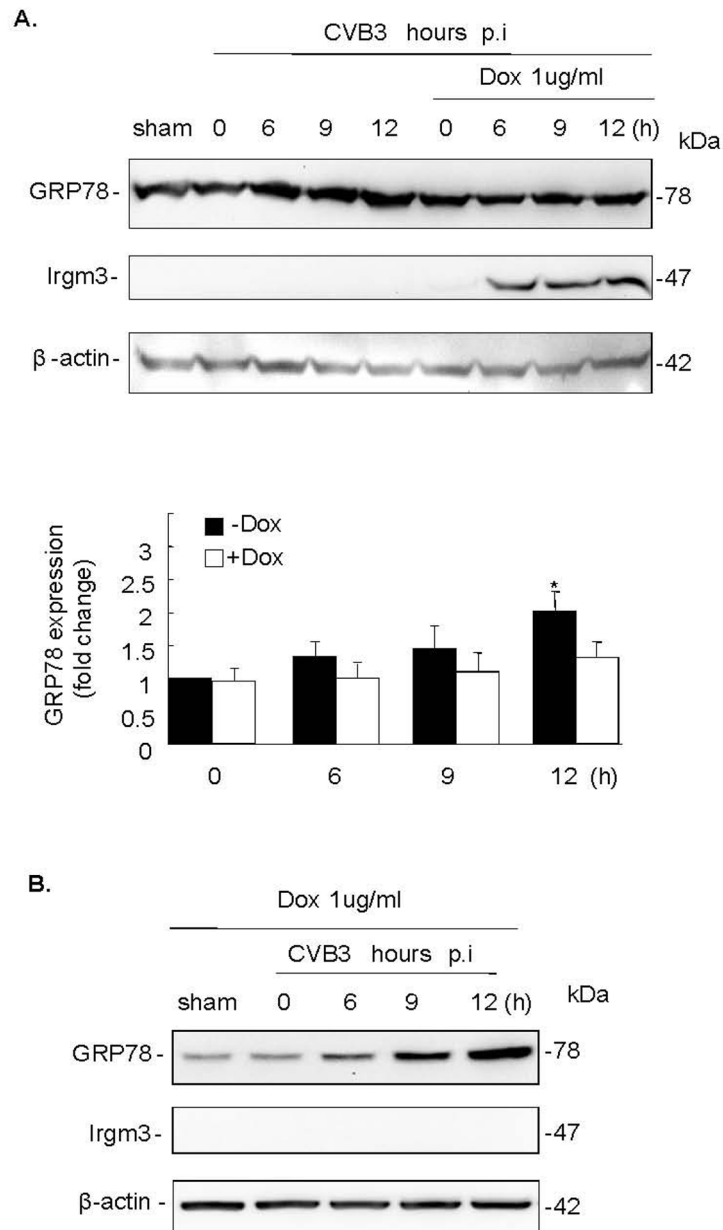
### Figure 3. Irgm3 inhibits the activation of ATF6 pathway of ER stress response

Tet-On/Irgm3 HeLa cells were induced with Dox and then treated with Tu or untreated as described in Fig. 2. Cell fractionation was performed, and cytosolic and nuclear fractions were collected separately. Proform and cleaved forms of ATF6 were detected in the cytosolic and nuclear fraction, respectively. Results were quantitated by densitometric analysis and normalized to the β-actin and histone-1 expression, respectively. Untreated controls were arbitrarily set to 1.0, and levels of ATF6 were calculated as fold increase compared to the control group. Data represents the mean ± S.E. from three independent experiments (\*p<0.05).



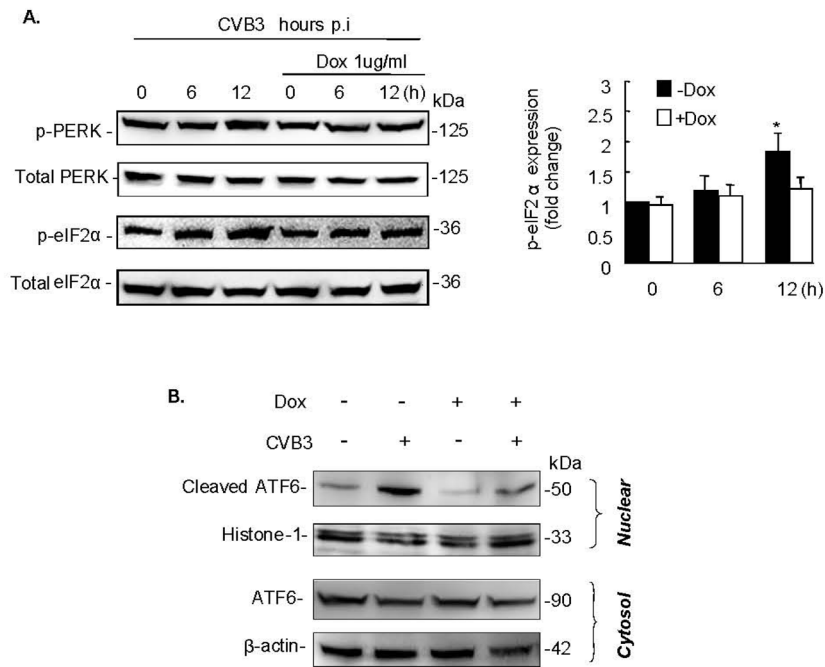


**Figure 4. Irgm3 expression inhibits the ER stress-mediated apoptotic response**  
 Tet-On/Irgm3 HeLa cells were induced with 1  $\mu$ g/ml Dox for 16 h and then treated with 1  $\mu$ g/ml Tu for 20 h or untreated. Cell lysates were harvested and subjected to immunoblot analysis with indicated antibodies.  $\beta$ -actin was used as the loading control.

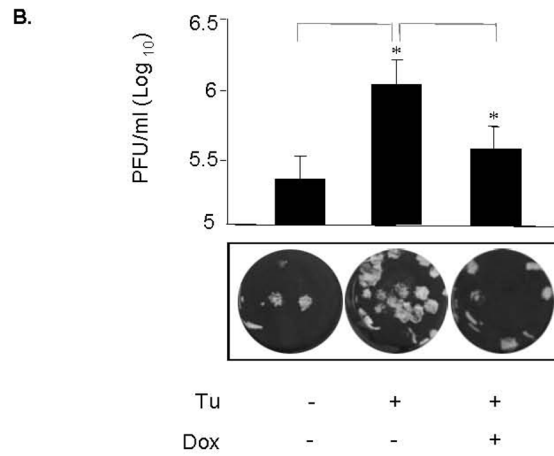
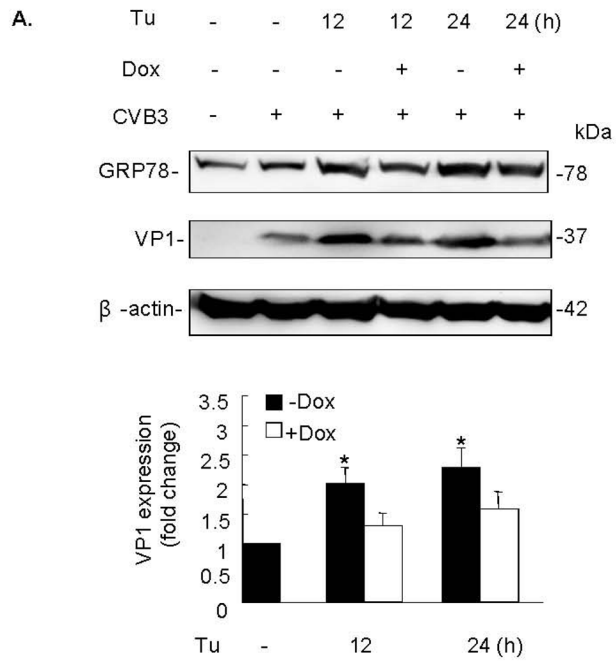


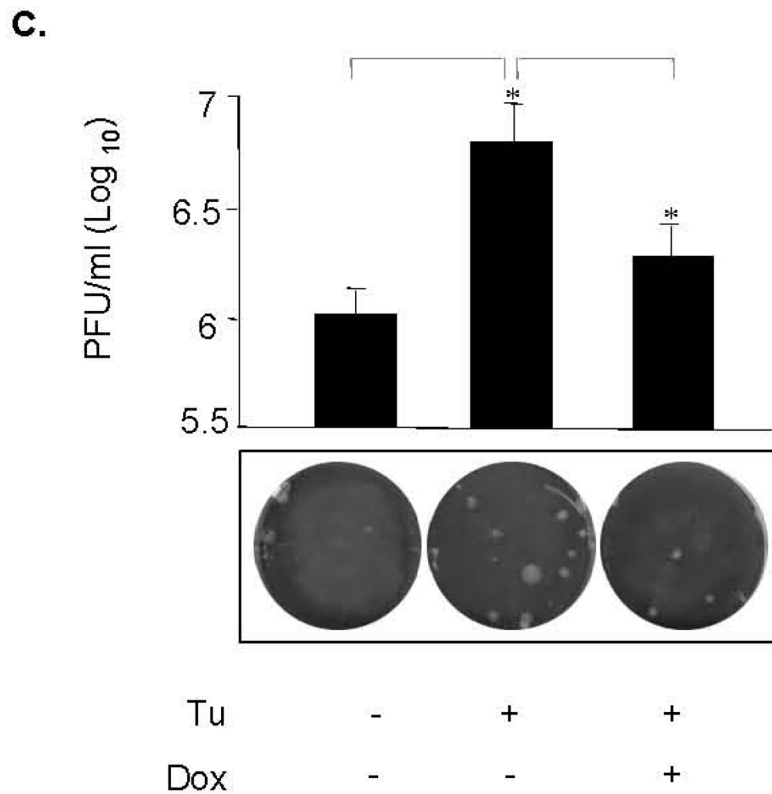
**Figure 5. Irgm3 inhibits the CVB3-induced upregulation of GRP78**

(A). Tet-On/Irgm3 HeLa cells were treated with 1  $\mu$ g/ml Dox for 20 h or untreated and then infected with CVB3 at 10 MOI or sham infected with PBS. Cell lysates were collected at the indicated time points pi and subjected to immunoblotting. GRP78 expression was quantitated by densitometric analysis and normalized to the  $\beta$ -actin expression. The value of GRP78 expression for the uninduced sample at 0 h was arbitrarily set to 1.0, and fold changes of other samples were calculated as compared to the control group. Data represents the mean  $\pm$  S.E. from three independent experiments (\* $p$ <0.05). (B). To confirm that vector or Dox itself has no effect on GRP78 expression, premade Tet-On HeLa cells were induced with Dox and then infected with CVB3 or sham-infected as described above. Cell lysates were collected at the indicated time points pi and subjected to immunoblotting for GRP78 and Irgm3 expression.



**Figure 6. Irgm3 inhibits the activation of PERK and ATF6 pathways during CVB3 infection**  
**(A).** Tet-On/Irgm3 HeLa cells were treated with 1  $\mu$ g/ml Dox for 20 h or untreated and then infected with CVB3 at 10 MOI. Cell lysates were collected at the indicated time points pi and subject to immunoblot analysis using indicated antibodies. The expression of p-eIF2  $\alpha$  were quantitated by densitometric analysis and normalized to the total eIF2  $\alpha$  expression. The value of p-eIF2  $\alpha$  for the uninduced control sample at 0 h was arbitrarily set to 1.0, and fold change of other samples were calculated based on the value of control. The data represents the mean  $\pm$  S.E. from three independent experiments (\* $p$ <0.05). **(B).** Cells were induced with Dox for 20 h and then infected with CVB3 at 10 MOI or sham-infected with PBS for 12 h. Cell fractions were prepared, and the proform and cleaved forms of ATF6 were detected in the nuclear and cytosolic fraction, respectively. Histone-1 and  $\beta$ -actin expression levels were used as loading controls.

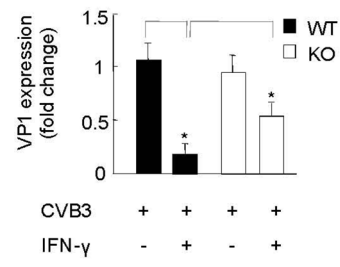
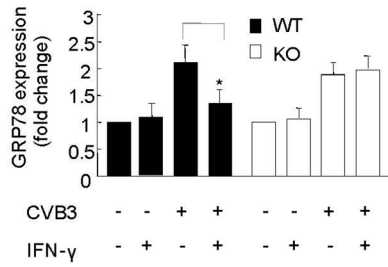
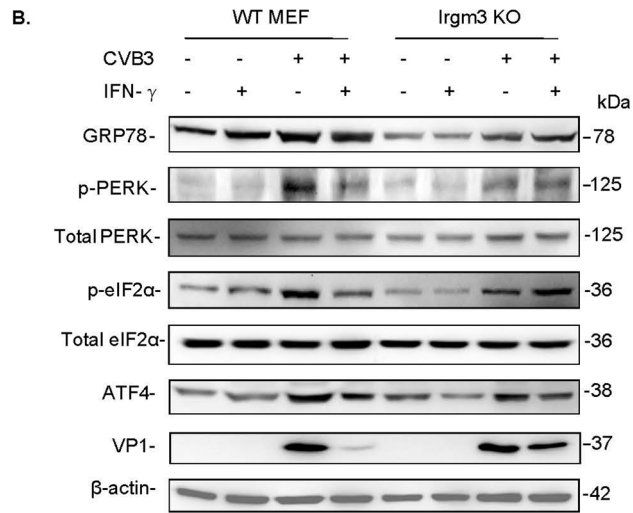
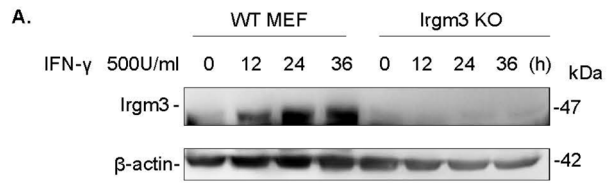


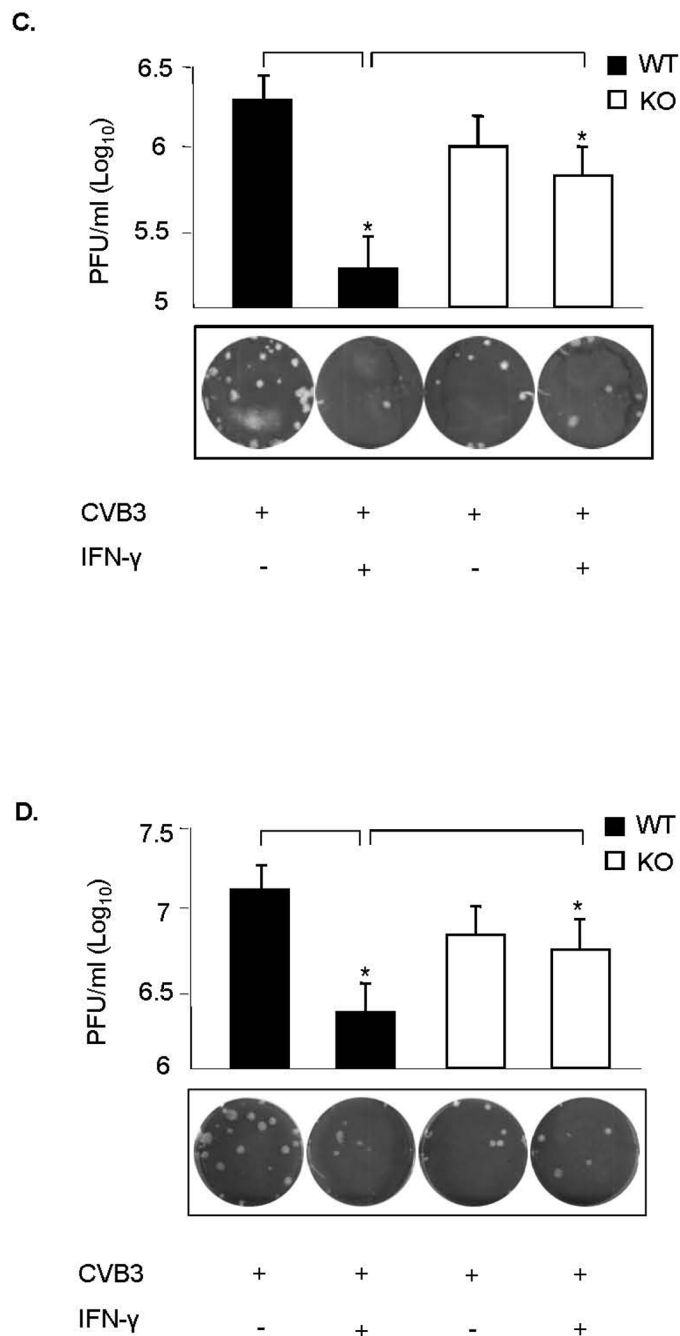


**Figure 7. Preemptive ER stress response induced by Tu facilitates viral protein synthesis and viral particle release**

Dox-induced or uninduced Tet-On/Irgm3 HeLa cells at 16 h post induction were treated with or without  $1\mu\text{g/ml}$  Tu for indicated hours and then infected with CVB3 at 10 MOI for 10 h. Cell lysates were collected and subjected to immunoblot analysis using indicated antibodies. CVB3 VP1 protein expression was quantitated by densitometric analysis and normalized to the  $\beta$ -actin expression. VP1 level of untreated cells was arbitrarily set to 1.0, and fold increase of other samples was calculated based on the untreated control. Data represents the mean  $\pm$  S.E. from three independent experiments ( $*p<0.05$ ) (A). Dox induced or uninduced Tet-On/Irgm3 HeLa cells were treated with or without  $1\mu\text{g/ml}$  Tu for 12 h and then infected with CVB3 for 10 h at 10 MOI. Supernatants only (B) and supernatants plus cells (C) from the cell cultures were collected respectively, and the pfu/ml was determined by plaque assays on HeLa cell monolayers. Data represents the mean  $\pm$  S.E from three independent experiments ( $*p<0.05$ ).



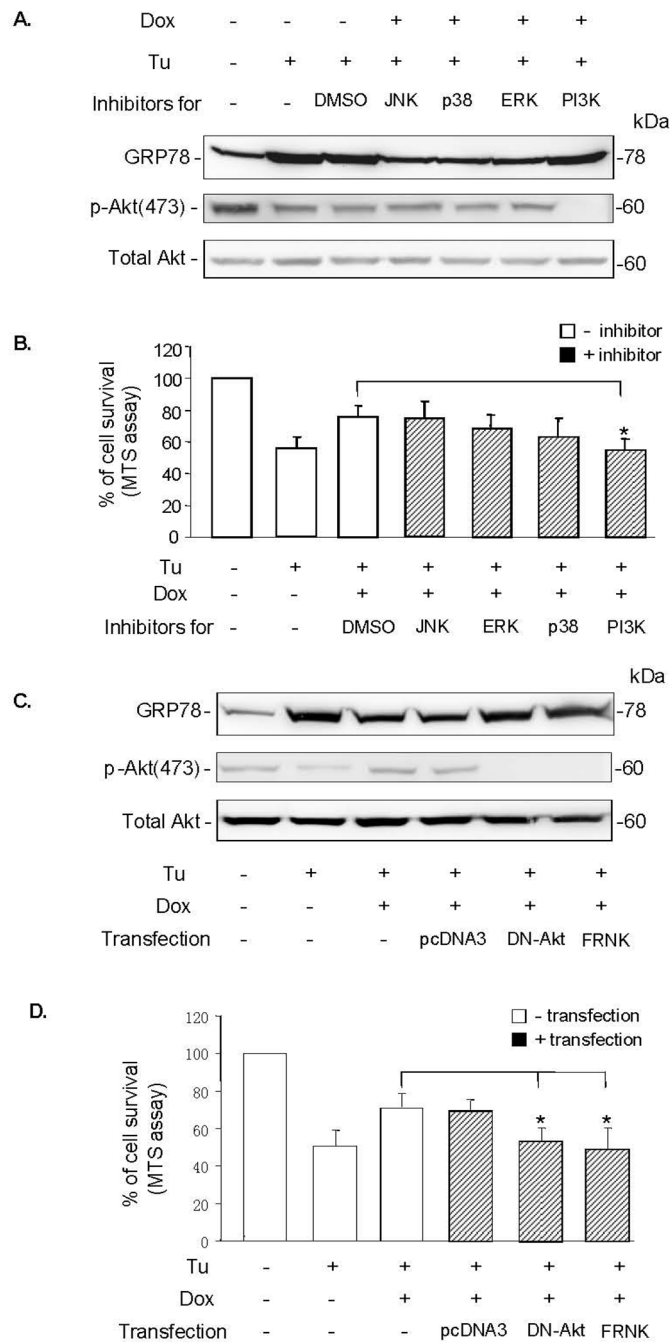




**Figure 8. Irgm3 relieves CVB3-induced ER stress response in mouse embryonic fibroblasts (MEF)**

WT MEF and Irgm3 KO MEF were treated with 500 U/ml IFN- $\gamma$  for indicated hours. Cell lysates were collected after treatment and Irgm3 expression was probed by immunoblotting (A). WT and KO MEF were infected with CVB3 at 40 MOI or sham-infected with PBS in the presence or absence of 500U/ml IFN- $\gamma$ . Cell lysates were harvested at 30 h pi and subjected to immunoblot analysis using indicated antibodies. GRP78 and VP1 expression were quantitated by densitometric analysis and normalized to the  $\beta$ -actin expression respectively. Untreated/sham-infected control from either cell line was arbitrarily set to 1.0, and fold increase of other samples were calculated based on the value of their respective

controls. Data represents the mean  $\pm$  S.E from three independent experiments (\* $p < 0.05$ ) (B). Supernatants only (C) and supernatants plus cells (D) from the cell cultures that were treated as described in (B) were collected respectively, and the pfu/ml was determined by plaque assays on HeLa cell monolayers. Data represents the mean  $\pm$  S.E from three independent experiments (\* $p < 0.05$ ).



**Figure 9. The relief of ER stress response by Irgm3 depends on the activation of PI3K/Akt pathway**

(A). Dox-induced or uninduced Tet-On/Irgm3 HeLa cells were treated either with vehicle (DMSO) or with specific inhibitors including PI3K inhibitor LY294002 at 10  $\mu$ M, ERK inhibitor U0126 at 20  $\mu$ M, p38 inhibitor SB203580 at 20  $\mu$ M, and JNK inhibitor SP600125 at 40  $\mu$ M. Cells were then treated with 1  $\mu$ g/ml Tu for 16 h. Cell lysates were harvested and subjected to immunoblot analysis. Total Akt was used as a loading control. (B). After cells were treated with inhibitors and Tu as described above, cell viability was measured by MTS assay. Absorbance values were corrected by subtracting the background reading. The value of MTS assay in untreated cells was defined as 100% survival (control). The cell viability of

other samples was expressed relative to the control. Data represents the mean  $\pm$  S.E from three independent experiments (\* $p < 0.05$ ). (C). Tet-On/Irgm3 HeLa cells were transiently transfected with a plasmid vector, or a plasmid encoding DN-Akt or FRNK using Lipofectamine 2000. At 24 h post transfection, cells were then treated with 1  $\mu\text{g/ml}$  Tu for 16 h and then subjected to immunoblot analysis. (D). Cells treated under the same conditions as described in (C) were also subjected to MTS assay. The value of cell viability of untreated cells was defined as 100% survival (control). The cell viability of other samples was expressed relative to the control. Data represents the mean  $\pm$  S.E from three independent experiments (\* $p < 0.05$ ).