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## CHARACTERIZATION OF THE ROLE OF CARMA3 IN ENDOPLASMIC RETICULUM STRESS-INDUCED NF- $\kappa$ B ACTIVATION

Zhicheng Zhou Mr

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TITLE OF THESIS

CHARACTERIZATION OF THE ROLE OF CARMA3 IN ENDOPLASMIC RETICULUM  
STRESS-INDUCED NF- $\kappa$ B ACTIVATION

By

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THESIS

Presented to the Faculty of  
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# CHARACTERIZATION OF THE ROLE OF CARMA3 IN ENDOPLASMIC RETICULUM STRESS-INDUCED NF- $\kappa$ B ACTIVATION

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## ABSTRACT

Endoplasmic reticulum (ER) stress-induced inflammation plays an important role in the progression of many diseases, such as type II diabetes, insulin resistance, cancers, and so on. NF- $\kappa$ B is believed to be a central regulator of ER stress-induced inflammation. However, studies on how ER stress induces NF- $\kappa$ B activation are limited and, in some cases, controversial. In the present study, we utilized two commonly used ER stress inducers, thapsigargin and tunicamycin, to study the mechanism. We found that two caspase-recruitment domain (CARD)-containing proteins, CARMA3 and BCL10, play a crucial roles on ER stress-induced NF- $\kappa$ B activation by regulating I $\kappa$ B $\alpha$  kinase activity. Consistently, we observed that a physiological ER stress inducer, hypoxia, could activate NF- $\kappa$ B in a CARMA3-dependent manner. Additionally, we showed that the activation of the UPR signaling pathways were intact in both CARMA3- and BCL10-deficient cells under ER stress. Together, this study provides insight into the mechanism of how ER stress induces NF- $\kappa$ B activation. It allows us to better understand ER stress-induced inflammation and develop the corresponding therapeutic interference to treat diseases.

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## CHAPTER 1: INTRODUCTION

### 1.1. ER Stress Signaling Pathway

In eukaryotic cells, the endoplasmic reticulum (ER) is an important organelle. It functions as the first step of the secretory pathways and the site of synthesis for ER proteins and proteins targeted for the Golgi compartment, endosomes, lysosomes, the plasma membrane, and the extracellular milieu (Back and Kaufman, 2012). As a major signal transducing organelle, the ER is highly adept at sensing and responding to changes in cell homeostasis (Zhang and Kaufman, 2008). ER stress is used to describe any stress in the ER. Among them, the most common stress observed is the accumulation of unfolded protein - induced the unfolded protein response (UPR), or excessive protein traffic caused by viral infection, which induces the ER overload response (EOR). These two cellular responses can further trigger activation of their downstream signaling cascades, which may play an important role in releasing and reducing cellular ER stress (Zhang and Kaufman, 2008).

In eukaryotic cells, ER stress-induced UPR is highly regulated by three different sensors: the PKR-like eukaryotic initiation factor 2 $\alpha$  kinase (PERK), the activating transcription factor-6 (ATF6), and the inositol requiring enzyme 1 (IRE1) pathways (Hotamisligil, 2010). In resting cells, the three proteins are endoplasmic reticulum (ER) membrane-anchored and interact with a chaperone called Bip/GRP78, which makes them “silenced”. In response to ER stress, Bip/GRP78 dissociates from them and binds to the unfolded proteins. Thereafter, the “free” PERK, ATF6, and IRE1 proteins are activated through different mechanisms and then initiate downstream signaling cascades.



In PERK pathway, the "free" PERKs can form homodimers and thus be activated. These activated forms of PERK specifically phosphorylates eukaryotic translational initiation factor 2 (eIF2 $\alpha$ ) on Ser-51, leading to the inactivation of this protein, which result in the blockade of global translation. The result is a decrease of nascent protein into the ER lumen and help ER release stress. Another signal event of the activation of PERK signal pathway is the increase in translation of ATF4, a member of the bZIP family of transcription factors. The increase of ATF4 enhances the expression of many downstream genes that are involved in amino acid biosynthesis, antioxidant stress responses, transport functions, and apoptosis. One of them is the transcription factor C/EBP homology (CHOP/GADD153), a classic ER stress marker (Verfaillie et al., 2010).

Similar to PERK activation, the "free" IRE1 undergoes homodimerization, which results in trans-autophosphorylation and production of mature XBP1 mRNA (spliced XBP1) (Calton et al., 2002). Then, mature XBP1 protein enters into nucleus and works as a transcription factor to increases the transcription of downstream genes required for ER/Golgi biogenesis, lipid synthesis, and ER-associated protein degradation (ERAD). One of them is Bip, a known ER chaperone and also a classic ER stress marker (Acosta-Alvear et al., 2007).

Another ER stress sensor is ATF6. Different from activation of PERK and IRE1, the "free" ATF6 transports from ER lumen to the Golgi apparatus, where it is cleaved by a couple of proteases. After that, the spliced ATF6 acts as a transcription factor and enters into the nucleus to enhance the transcription of genes that help to release ER stress, such as GRP94, BiP, and calreticulin (Yoshida et al., 1998) (Fig.1).

In summary, activation of these three signaling pathways may lead to the following events: (1) translational attenuation, (2) increase of transcription of ER chaperone proteins, and (3) enhancement of ER-related protein degradation. These events facilitate cells to decrease the influx of nascent polypeptides into the ER and thus alleviate ER stress. However, in some cases, the cell dies, if the ER stress signal is too strong and persistent. For example, the free IRE1 can interact with pro-apoptotic BAX and BAK proteins, which enhance the permeability of the mitochondrial membrane and release of cytochrome C (Hetz et al., 2006). Furthermore, the activated IRE1 can also interact with caspase 12 through the tumor necrosis factor receptor-associated factor (TRAF2), and therefore lead to apoptosis (Yoneda et al., 2001). Additionally, the ER stress marker, CHOP has been reported to mediate ER-stress induced apoptosis in human carcinoma cells by enhancing death receptors DR5 expression (Yamaguchi and Wang, 2004).

Compared to studies of the UPR, studies of the EOR are limited, although the EOR can be easily elicited by overexpression or accumulation of ER membrane-localized proteins. Many viruses appear to cause the EOR by accumulation of viral proteins in the ER, for example, the influenza hemagglutinin and adenovirus E3/19K membrane protein (Santoro et al., 2003). In eukaryotic cells, ER stress induced by the EOR can trigger  $\text{Ca}^{2+}$  release from the ER lumen to cytoplasm. Then, the cytosolic  $\text{Ca}^{2+}$  is taken up by mitochondria, and thus inducing production of reactive oxygen species (ROS) (Pahl and Baeuerle, 1997). The  $\text{Ca}^{2+}$  and ROS serve as crucial second messengers involved in the activation of many other signaling pathways, including, the nuclear factor-kappaB (NF- $\kappa$ B) signaling pathway (Mazars et al., 2010).

## 1.2. NF- $\kappa$ B Activation

NF- $\kappa$ B is a family of transcription factors that control the expression of various important proteins involved in inflammation, immune response, cell growth, apoptosis, and differentiation (Hayden and Ghosh, 2004; Natoli, 2010). In resting cells, NF- $\kappa$ B is associated with I $\kappa$ B proteins that block the NF- $\kappa$ B nuclear localization domains, and thereby preventing translocation of NF- $\kappa$ B into the nucleus. Classic NF- $\kappa$ B activation is mediated by a wide variety of receptors, including receptors for proinflammatory cytokines, such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), toll-like receptors (TLRs), T cell receptors (TCRs), growth factor receptors, such as epithelial growth factor receptor family members, and G protein-coupled receptors. Upon receipt of appropriate signals, many signaling complexes are activated, leading to activation of the I $\kappa$ B kinase (IKK) complex. Activation of the IKK complex results in I $\kappa$ B $\alpha$  phosphorylation, ubiquitination, and degradation, which frees the NF- $\kappa$ B dimers. Then, the free NF- $\kappa$ B goes into the nucleus and initiates transcription of its target genes (Hayden and Ghosh, 2004; Perkins, 2007).

More recently, tremendous progress has been made in revealing different receptors – induced NF- $\kappa$ B signaling pathways, such as TNF receptor, TLR, TCR, and so on. However, the mechanistic details of activation of the IKK complex in the context of different situations still remain to be fully elucidated. The scaffold molecule is believed to act as a receptor-and tissue-specific roles in mediating NF- $\kappa$ B signaling. Studies in our laboratory and others have demonstrated that a scaffold protein, CARMA1, works in the downstream of protein kinase

C theta (PKC $\theta$ ) and is required for T cell receptor (TCR) and B cell receptor (BCR)-induced NF- $\kappa$ B activation (Gaide et al., 2002; Pomerantz et al., 2002; Wang et al., 2002). In response to activation of TCR and BCR, CARMA1 is phosphorylated by PKC $\theta$  and PCK $\beta$ . After its activation, CARMA1 recruits its binding partners, such as B-cell lymphoma/leukemia 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to form the CARMA1-BCL10-MALT1 signalosome (Che et al., 2004; Gaide et al., 2002; Hara et al., 2004; Wang et al., 2004). Formation of this signalosome has been shown to play an essential role in mediating IKK activation through an ubiquitination-dependent pathway (Ruefli-Brasse et al., 2003; Ruland et al., 2003; Sun et al., 2004; Zhou et al., 2004). CARMA1 has two homolog proteins, CARMA2 and CARMA3. These three proteins shared the same motifs, including an N-terminal caspase-recruitment domain followed by a coiled-coil domain, a PDZ domain, an SH3 domain, and guanylate kinase-like domain. But they exhibit distinct patterns of tissue distribution. Specifically, CARMA1 is expressed in hematopoietic tissues; CARMA2 is expressed only in the placenta; and CARMA3 is expressed in all non-hematopoietic tissues (Bertin et al., 2001; Gaide et al., 2001; McAllister-Lucas et al., 2001; Wang et al., 2001). This distinct tissue distribution suggests that CARMA family members may play similar roles in homologous signaling pathways but in different tissues. Consistent with this concept, the role of CARMA3 in mediating G protein-coupled receptor is similar to that of CARMA1 in TCR signaling pathway. After activation, CARMA3 recruits downstream protein BCL10 to regulate K63-linked ubiquitination of the IKK complex. In addition, CARMA3-mediated NF- $\kappa$ B activation

can be blocked by PKC inhibitors, which suggests that PKC isoforms may exert an important effect on activating CARMA3 just as they are important in activating CARMA1 (Blonska and Lin, 2011).

### 1.3. ER Stress-Induced NF- $\kappa$ B Activation

ER-stress induced inflammation is an efficient way to communicate between the stressed cells and other cells in a certain tissue or in the organism as a whole. It is well established that ER stress-induced signaling pathways are highly associated with production of many proinflammatory molecules (Li et al., 2005). All of the three main signaling pathways of UPR have been shown to mediate many proinflammatory transcriptional programs. Among them, NF- $\kappa$ B is a central mediator of proinflammatory pathways.

However, results from the studies of NF- $\kappa$ B activation under ER stress condition are limited and, in some cases, controversial. On the one hand, the initial studies provided evidence for activation of NF- $\kappa$ B by all the three signaling pathways of UPR through different mechanisms. To be specific, following ER stress, the activated PERK-eIF2 $\alpha$  arm causes translational arrest, which will decrease the production of I $\kappa$ B $\alpha$  and NF- $\kappa$ B proteins. This leads to the change in the ratio of NF- $\kappa$ B dimmers to I $\kappa$ B $\alpha$  in terms of protein level since the half-life of I $\kappa$ B $\alpha$  protein was much shorter than NF- $\kappa$ B proteins in resting cells. Thus, there are not enough I $\kappa$ B $\alpha$  proteins to block NF- $\kappa$ B dimmers in the cytoplasm. Therefore, the “free” NF- $\kappa$ B dimmers enter into the nucleus and initiate the expression of downstream genes. Additionally, ER stress can also cause activation of IRE1 $\alpha$ , which can

recruit and activate the IKK complex in a tumor necrosis factor (TNF)- $\alpha$ -receptor-associated factor 2 (TRAF2)-dependent manner. The activated IKK can further phosphorylate I $\kappa$ B $\alpha$  on Ser 32 and 34 and lead to its degradation by proteasomes, and thereby freeing NF- $\kappa$ B dimers (Hu et al., 2006; Jiang et al., 2003; Kaneko et al., 2003). Similar to IRE1 $\alpha$ -IKK arm, the ATF6-mediated NF- $\kappa$ B activation also requires the IKK complex but through a different mechanism. The activated ATF6 results in an activation of the AKT-IKK pathway (Nakajima et al., 2011a; Yamazaki et al., 2009)(Fig. 2). On the other hand, mounting evidence suggests that ER stress sometimes can inhibit NF- $\kappa$ B activation. For example, ER stress caused by proteasome inhibition suppresses NF- $\kappa$ B activation independent of I $\kappa$ B degradation. In this report, ER stress was found to be able to transiently upregulate the transcription factor C/EBPB, which triggers sustained expression of its downstream genes coding for liver-enriched inhibitory protein (LIP) and liver activating protein (LAP). These two proteins play a key role in suppressing NF- $\kappa$ B activation (Nakajima et al., 2011b; Pepys and Hirschfield, 2003). These seemingly contradictory facts suggest a complicated role of ER stress in NF- $\kappa$ B activation. It may depend on cell type and ER stress inducers. More studies are needed to elucidate the role of ER stress in NF- $\kappa$ B activation and inflammation.

#### 1.4. ER Stress Inducers: Thapsigargin (Tg) and Tunicamycin (Tuni)

Thapsigargin is a natural chemical extracted from a plant named *Thapsia garganica*. It is a well-established inhibitor of a class of enzymes called sarco-endoplasmic reticulum

$\text{Ca}^{2+}$ -ATPases (SERCA) in mammalian cells. Thapsigargin can raise cytosolic calcium concentration in an inositol triphosphate (IP3)-independent manner. Instead, it blocks the activity of SERCA to pump calcium from the cytosol of the cell to the lumen of the ER and causes calcium storage in the ER to become depleted. Over the past thirty years, thapsigargin has been a powerful tool to elucidate the mechanism of intracellular  $\text{Ca}^{2+}$  signaling pathway (Treiman et al., 1998). In addition, the change of calcium homeostasis in the ER can lead to malfunction of ER chaperones that are involved in protein folding and quality control system. Therefore, thapsigargin can also be used to induce ER stress in cells and to study ER stress-related signaling pathways (Torres et al., 2011).

Another ER stress inducer is tunicamycin, a mixture of homologous antibiotics and antiviral compounds. It is originally isolated from several bacteria, including *Streptomyces clavuligerus* and *Streptomyces lysosuperficus*. It is widely used as an inhibitor of UDP-N-acetylglucosamine—dolichyl-phosphate N-acetylglucosaminephosphotransferase, an enzyme in the ER that catalyzes the first committed step of N-linked glycosylation of protein biosynthesis in cells. Then, tunicamycin can cause extensive protein misfolding in the ER, which leads to ER stress (Bassik and Kampmann, 2011).

Additionally, it has been shown that tunicamycin can also raise cytosolic calcium concentration by releasing  $\text{Ca}^{2+}$  from the ER. However, the mechanism is unclear. It may be due to  $\text{Ca}^{2+}$  leak channels (Buckley and Whorton, 1997). Since both thapsigargin and tunicamycin can increase cytosolic calcium after treatment, it suggests that intracellular  $\text{Ca}^{2+}$  signaling pathway may contribute to ER stress response. However, the studies in this field is quite limited. Further studies are warranted to uncover the mechanisms behind this.

### 1.5. Hypoxia-Induced ER Stress and NF- $\kappa$ B

Hypoxia is defined as a pathological condition of the lack of oxygen in cells, tissues, or the body. Also, a mismatch between oxygen demand and supply may lead to a hypoxic condition in cells. Under hypoxic conditions, cells initiate a series of cellular and molecular responses to adapt to the environment with inadequate oxygen (Semenza, 2008).

The transcriptional factor hypoxia-inducible factor (HIF) is considered as the most important regulator of the complicated cellular response to hypoxia (Semenza, 2008). Under normoxic conditions (no hypoxia), HIF-1 $\alpha$  subunits are hydroxylated at the conserved proline residues by a class of prolyl hydroxylase domain (PHD) enzymes and the factor inhibiting HIF (FIH). The hydroxylated HIF-1 $\alpha$  proteins are recognized and bind to the E3 ubiquitin ligase von Hippel-Lindau complex, leading to degradation of the protein by proteasomes. Therefore, HIF-1 $\alpha$  subunits have a short half-life and thus low transcriptional activity in normoxic conditions. In hypoxia, the HIF-1 $\alpha$  subunit becomes stable since the prolyl-hydroxylase is inhibited. The stabilized HIF-1 $\alpha$  proteins will form heterodimers with constitutively expressed HIF-1 $\beta$  and up-regulate the expression level of several downstream genes, such as vascular endothelial growth factor. These genes together have an important impact on helping cell survival in hypoxia (Fandrey et al., 2006; Kaelin and Ratcliffe, 2008).

Although HIF signaling pathway is believed to be the master regulator of cellular response to hypoxia, other signaling pathways may also contribute to it. Two major ones of them are hypoxia-induced ER stress and NF- $\kappa$ B.



Hypoxia is a physiological ER stress inducer. It leads to protein misfolding and accumulation in the ER. In eukaryotic cells, the ER functions as the first station of secretory pathways and the site of synthesis for all the secreted and transmembrane proteins. Different from the cytosolic proteins, most secreted proteins need a series of well-organized post-translational modifications, including disulfide bonding, glycosylation, and et al. These modifications sometimes determine the “native” or the “folded” state of proteins. Any disruption of these post-translational modifications would result in generation of misfolded proteins and protein accumulation in the ER. Under hypoxic conditions, if some proteins do not fold correctly, they have to accumulate in the ER and cause ER stress. For example, Ero1, which is transcriptionally induced by HIF in hypoxia, cannot fold correctly without oxygen (Feldman et al., 2005).

Hypoxia-induced ER stress plays an essential role under many physiological and pathological conditions. For example, in hypoxic avascular tissues, the stabilized HIF-1 $\alpha$  collaborates with the ER stress signaling pathways to maintain proper growth-plate development. Specifically, the PERK-EIF2 $\alpha$ -ATF4 branch is activated under hypoxia, and thus positively regulates chondrocyte proliferation and differentiation (Wang et al., 2009). Additionally, the evidence from both experimental and clinical studies have indicated an important role of hypoxia-induced ER stress in solid tumors (Feldman et al., 2005). Therefore, more understanding of hypoxia-induced ER stress will help us to develop new therapeutic strategies for these disease.

Hypoxia is also a physiological NF- $\kappa$ B inducer. The original discovery that NF- $\kappa$ B is activated in hypoxia was made in 1994. In this study, it was found that hypoxia can cause

I $\kappa$ B $\alpha$  degradation through the phosphorylation of I $\kappa$ B $\alpha$  on tyrosine residues (Koong et al., 1994). Inconsistent with earlier studies, a recent study has shown that hypoxia-induced NF- $\kappa$ B activation is in a non-I $\kappa$ B $\alpha$  degradation manner. It was found that hypoxia also causes tyrosine phosphorylation of I $\kappa$ B $\alpha$  and but prevents it from degradation. Surprisingly, this tyrosine phosphorylation is IKK dependent. This result suggests a novel mechanism of NF- $\kappa$ B activation under hypoxic conditions. Moreover, it was found that NF- $\kappa$ B activation requires calcium as well as the activation of calcium/calmodulin-dependent kinase 2 (CaMK2II) (Culver et al., 2010). The difference in the results between the two studies may be due to cell type and different hypoxic conditions being used. It suggests the complexity of hypoxia-induced NF- $\kappa$ B signaling pathway. Considering the importance of both hypoxia and NF- $\kappa$ B signaling pathway under physiological and pathological conditions, it would be very interesting to see more coming studies in this field.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1. Antibodies, Plasmids, and Reagents

Phosphorylation-specific antibodies, including I $\kappa$ B $\alpha$  (9246L), IKK $\alpha/\beta$ , PERK, CHOP, Bip, and eIFII $\alpha$ , were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against I $\kappa$ B $\alpha$  (371, clone C21), IKK $\alpha/\beta$  (7607, clone H-470), and actin (8432, clone C-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotide probes for NF- $\kappa$ B (E3291) and OCT-1 (E3241) were purchased from Promega (Madison, WI). Thapsigargin (T9033) and tunicamycin were obtained from Sigma-Aldrich (St. Louis, MO) and Enzo bichem respectively. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) was purchased from Endogen (Woburn, MA). TRIzol (15596-026) and Superscript III First-Strand Synthesis system (18080051) were obtained from Invitrogen (Carlsbad, CA) and DNase I kits (10104159001) were purchased from Roche Applied Science (Indianapolis, IN). Two times SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA).

### 2.2. Cell Cultures

CARMA3- and BCL10- wildtype (WT) and deficient mouse embryonic fibroblasts (MEF) cells were prepared as described previously (Grabiner et al., 2007). Cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Human hepatoma Huh-7 cells were cultured under the same conditions. Cells were grown in 8.5% CO<sub>2</sub> at 37°C and passaged every three days.

### 2.3. Electrophoretic Mobility Shift Assay (EMSA)

EMSA experiments were performed as described previously (Grabiner et al., 2007). Briefly,  $1 \times 10^6$  to  $2 \times 10^6$  cells were stimulated for appropriate times with various agents, and then nuclear extracts were prepared. Then  $1 \times 10^5$  cpm of  $^{32}\text{P}$ -labeled probes for NF- $\kappa\text{B}$  and OCT-1 were incubated with nuclear extracts (5–10  $\mu\text{g}$ ) at room temperature for 15 minutes. The mixed samples were separated on a native Tris-borate-EDTA polyacrylamide gel. The gels were dried at  $80^\circ\text{C}$  for one hour, and then exposed to X-ray film.

### 2.4. Western Blotting

Cells were lysed in a buffer containing 50 mM HEPES (pH 7.4), 250mM NaCl, 1% nonidet P-40, 1 mM EDTA, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 1mM PMSF, 1mM dithiothreitol, and a protease inhibitor cocktail (Roche Diagnostics). The cell lysates were subjected to SDS-PAGE and western blotting or immunoprecipitated with various antibodies.

### 2.5. Quantitative RT-PCR (qRT-PCR) and Analysis of mRNA Cleavage of XBP-1

mRNA isolation for all procedures was performed using TRIzol reagent according to the manufacturer's instructions. RT was performed using the SuperScript III First-Strand Synthesis System with 2  $\mu\text{g}$  of total RNA and oligo dT (50  $\mu\text{M}$ ). One percent of the resulting cDNA was used for PCR with gene-specific oligonucleotide primers (10 pM each).

Analysis of mRNA cleavage of XBP-1 was performed using MangoTaq™ DNA polymerase. Forward and reverse primers, respectively, were mouse xbp1: 5'-AAACAGAGTAGCAGCGCAGACTGC-3' and 5'-

TCCTTCTGGGTAGACCTCTGGGAG-3'. PCR conditions were described previously (Calfon et al., 2002). RT-PCR products were resolved in 2.5% agarose gels, stained with ethidium bromide, and scanned under ultraviolet (UV) light using an Alpha Imager.

## 2.6.Lentivirus Infection for shRNA Knockdown

Lentiviruses for CARMA3 and BCL10 were generated as described previously (Jiang et al., 2011). Briefly, virus was generated by transfecting HEK293T with packaging vectors encoding VSV-G and  $\Delta$ VPR using the calcium precipitation method. Twelve hours after transfection, the supernatant was changed with the fresh one. Twenty-four hours after transfection, the supernatant was collected from the cells, spun at 2000  $\times$ g for 10 minutes to pellet any cell debris, and then applied directly to target cells. One day after infection, cells were placed under the selection with media containing puromycin, and resultant pools were used for experiments.

## 2.7.Hypoxia Induction

Hypoxia induction was performed as previously described (Culver et al., 2010). Briefly, cells were incubated in 1%O<sub>2</sub> levels in an InVivo 300 hypoxia workstation (Ruskin, United Kingdom). Before experiments, the medium was replaced with a hypoxia-equilibrated medium (the medium was equilibrated in the hypoxia workstation for overnight before being added to the cells) in the workstation. Cells were exposed to Hypoxia for indicated time points. After experiments, cells were lysed on ice immediately for extraction of protein to avoid reoxygenation.

## CHAPTER 3: RESULTS

### 3.1. CARMA3 and BCL10 Contribute to Thapsigargin-Induced NF- $\kappa$ B Activation.

Previous studies from our lab and others showed that the CARMA3-BCL10-MALT1(CBM) signalosome is essential for NF- $\kappa$ B activation induced by some G protein-coupled receptors and receptor tyrosine kinases (Grabiner et al., 2007; Klemm et al., 2007; Mahanivong et al., 2008; McAllister-Lucas et al., 2007). To determine whether CARMA3 plays an important role in ER stress-induced NF- $\kappa$ B activation, we first used the ER stress inducer thapsigargin to treat WT and CARMA3-deficient MEF cells. We found that thapsigargin effectively induced NF- $\kappa$ B activation in a dose-dependent manner in WT cells, but this activation was defective in CARMA3-deficient cells (Fig. 3A). Next, we decided to test NF- $\kappa$ B activation in MEFs at different time points after exposure to 2  $\mu$ M thapsigargin. As expected, we observed that thapsigargin also activated the NF- $\kappa$ B signaling pathway in a time-dependent manner after treatment in WT cells, but similarly this activation was defective in CARMA3-deficient cells. In addition, consistent with previous studies, treatment of CARMA3-deficient MEFs with the PKC agonist, phorbol-12-myristate-13-acetate (PMA), failed to induce NF- $\kappa$ B activation. In contrast, TNF-induced NF- $\kappa$ B activation was not significantly affected by the absence of CARMA3 (Fig. 3B). This evidence suggests that CARMA3 contributes to thapsigargin-induced NF- $\kappa$ B activation.

It has been established that CARMA3 functions together with BCL10 and MALT1 to form a scaffold complex, which triggers polyubiquitination of one or more unknown proteins that associate with NEMO possibly by linking NEMO to TRAF6 (Grabiner et al., 2007). To

examine whether BCL10 aids in thapsigargin-induced NF- $\kappa$ B activation, WT and BCL10-deficient MEFs cells were stimulated with thapsigargin for indicated time periods. Similar to what we observed for CARMA3-deficient MEFs, BCL10-deficient exhibited defective NF- $\kappa$ B after thapsigargin treatment compared to that of WT MEF cells (Fig. 3C). These findings suggest that the CARMA3-BCL10 signalosome exerts an important effect on ER stress-induced NF- $\kappa$ B activation. To examine if this is also true in other cell types, we knocked down CARMA3 and BCL10 expression by shRNA in Huh7 hepatoma cells and A431 cells. PMA and TNF $\alpha$  herein were used as controls. As expected, with CARMA3 and BCL10 shRNA, Huh7 cells displayed a reduced NF- $\kappa$ B activation after thapsigargin and PMA treatment but not TNF treatment (Fig. 3D). These data indicate that the knockdown efficiency of CARMA3 and BCL10 in Huh7 cells is good, and CARMA3 and BCL10 facilitate thapsigargin-induced NF- $\kappa$ B activation in Huh7 cells. We found that knockdown of CARMA3 also reduced NF- $\kappa$ B activation in A431 cells after exposure to thapsigargin (data not shown). Together, these findings suggest that CARMA3 and BCL10 play an important role in thapsigargin-induced NF- $\kappa$ B activation.

### 3.2.CARMA3-Regulated IKK Complex Activation Following Thapsigargin Treatment Is Dependent on IKK $\alpha/\beta$ Phosphorylation.

To decipher the role of CARMA3 and BCL10 in thapsigargin-induced NF- $\kappa$ B activation, we stimulated WT, CARMA3- and BCL10-deficient MEF cells with 2 $\mu$ M thapsigargin. At different time points, we examined whether phosphorylation of IKK  $\alpha/\beta$  and P65 and degradation of I $\kappa$ B $\alpha$  were defective in different cell types after treatment.

Consistent with the previous EMSA data, we found that thapsigargin could robustly induced I $\kappa$ B $\alpha$  decreased in WT, but not CARMA3- and BCL10-deficient cells (Fig. 4, middle panel). Also, P65 phosphorylation was partially defective in CARMA3- and BCL10-deficient cells. Since P65 phosphorylation at Ser 536 and degradation of I $\kappa$ B $\alpha$  have been linked to activation of the IKK complex, we further examined whether IKK $\alpha$ / $\beta$  was phosphorylated using antibodies against phospho-Ser176/180 of IKK $\alpha$  and phospho-Ser 177/181 of IKK $\beta$ . Similar to what we found before, IKK $\alpha$ / $\beta$  phosphorylation induced by thapsigargin was defective only in CARMA3- and BCL10-deficient cells (Fig. 4, top panel). Collectively, these data indicate that the IKK complex activation depends on CARMA3 and BCL10 in thapsigargin-induced NF- $\kappa$ B activation.

### 3.3.CARMA3 and BCL10 Contribute to Tunicamycin-Induced NF- $\kappa$ B Activation.

It has already been shown that CARMA3 and BCL10 contribute to thapsigargin-induced NF- $\kappa$ B. To further investigate the requirement of BCL10 and CARMA3 for NF- $\kappa$ B signaling pathway under ER stress, another ER stress inducer tunicamycin was used to treat WT, CARMA3-, and BCL10-deficient MEFs. These two drugs are most widely used to induce ER stress and to study ER stress signaling pathways (Samali et al., 2010). Unlike thapsigargin, which disturbs normal ER function by removing Ca<sup>2+</sup> from the organelle, tunicamycin induces ER stress by blocking the synthesis of all N-linked glycoproteins (N-glycans) (Han et al., 2008). We treated WT, BCL10-, and CARMA3-deficient MEF cells with 2 $\mu$ M thapsigargin, 2 $\mu$ g/ml tunicamycin, and 10ng/ml TNF, respectively. Here, thapsigargin and TNF worked as controls. Similar to thapsigargin, tunicamycin induced



robust NF- $\kappa$ B activation only in WT but not in BCL10- and CARMA3-deficient MEFs cells (Fig. 5A). These results suggest that ER stress-induced NF- $\kappa$ B activation depends on BCL10 and CARMA3.

To further confirm these results, we stimulated WT, BCL10-, and CARMA3-deficient MEF cells with 2  $\mu$ g/ml tunicamycin. At different time points, we examined whether P65 phosphorylation was defective in CARMA3-deficient MEF cells after treatment. We observed that P65 phosphorylation was defective in CARMA3-deficient MEF cells (Fig. 5B). These suggest that CARMA3 aids in tunicamycin-induced NF- $\kappa$ B activation.

#### 3.4.CARMA3 Contributes to Production of ER Stress-Induced Proinflammatory Cytokines in MEF Cells.

It is well established that NF- $\kappa$ B is one of the central mediators of proinflammatory pathways (Garg et al., 2012). Therefore, we examined whether CARMA3 facilitates production of ER stress-induced proinflammatory cytokines. To test this hypothesis, we stimulated WT and CARMA3-deficient MEF cells with thapsigargin and tunicamycin, respectively. 8 hours after stimulation, we collected cells and detected production of proinflammatory cytokines, IL6, TNF, and IL-1 $\beta$ , by real-time quantitative PCR. We found that the mRNA level of these cytokines was decreased in CARMA3-deficient cells compared to WT cells (Fig. 6). The result suggests that CARMA3 contributes to production of ER stress-induced proinflammatory cytokines.

#### 3.5.CARMA3 and BCL10 Are Not Required for the UPR Signaling.

To decipher the mechanism of how CARMA3 and BCL10 function in ER stress-induced NF- $\kappa$ B activation, we decided to study activation of all three branches of the UPR in CARMA3-deficient cells under ER stress. First, we examined activation of the PERK-EIF2 $\alpha$  arm of the UPR by detecting phosphorylation of PERK and EIF2 $\alpha$  in WT and BCL10- and CARMA3-deficient cells after thapsigargin treatment (Zhong et al., 2012). In Fig. 7, we found that activation of the PERK-EIF2 $\alpha$  was not defective in BCL10- and CARMA3-deficient cells compared to their corresponding WT cells. Next, we examined activation of the IRE1 $\alpha$ -XBP of the UPR by detecting the cleavage of xbp1 pre-mRNA in different MEF cells (Cnop et al., 2012). We found that there was no defective cleavage of xbp1 pre-mRNA in both WT and CARMA3-deficient MEF cells after treatment of both thapsigargin and tunicamycin, suggesting that activation of the IRE1 $\alpha$ -XBP1 of the UPR was intact in CARMA3-deficient MEF cells under ER stress (Fig. 8). Considering that two of the three branches of the UPR signaling pathways were intact in CARMA3-deficient cells, we hypothesized that activation of all UPR signaling pathways is intact in BCL10- and CARMA3-deficient cells. Therefore, we tested this hypothesis by detecting accumulation of BIP and CHOP in different MEF cells after both thapsigargin and tunicamycin treatment. We found that the level of BIP and CHOP is comparable between WT and knockout cells at each time point, which suggests that all the three UPR signaling pathways are intact in CARMA3- or BCL10-deficient cells (Fig. 9).

### 3.6. CARMA3 Contributes to Hypoxia-Induced NF- $\kappa$ B.

Although our data suggests that ER stress induced NF- $\kappa$ B depends on CARMA3 and BCL10, the whole study relied too much on thapsigargin and tunicamycin, two chemicals extracted from plants and bacteria, respectively (Treiman et al., 1998). Thus, we decided to add a physiological ER stress inducer, hypoxia, into our story. Since hypoxia can induce activation of both ER stress and NF- $\kappa$ B related signaling pathways specifically in a  $\text{Ca}^{2+}$ -dependent manner, we hypothesized that CARMA3 may have an impact on hypoxia-induced NF- $\kappa$ B. In order to test this hypothesis, we initiated hypoxia in WT and CARMA3-deficient cells and detected the NF- $\kappa$ B activation using EMSA. We observed that there is a defective NF- $\kappa$ B activation in CARMA3-deficient cells compared to that of WT MEF cells, suggesting that CARMA3 contributes to hypoxia-induced NF- $\kappa$ B (Fig. 10).

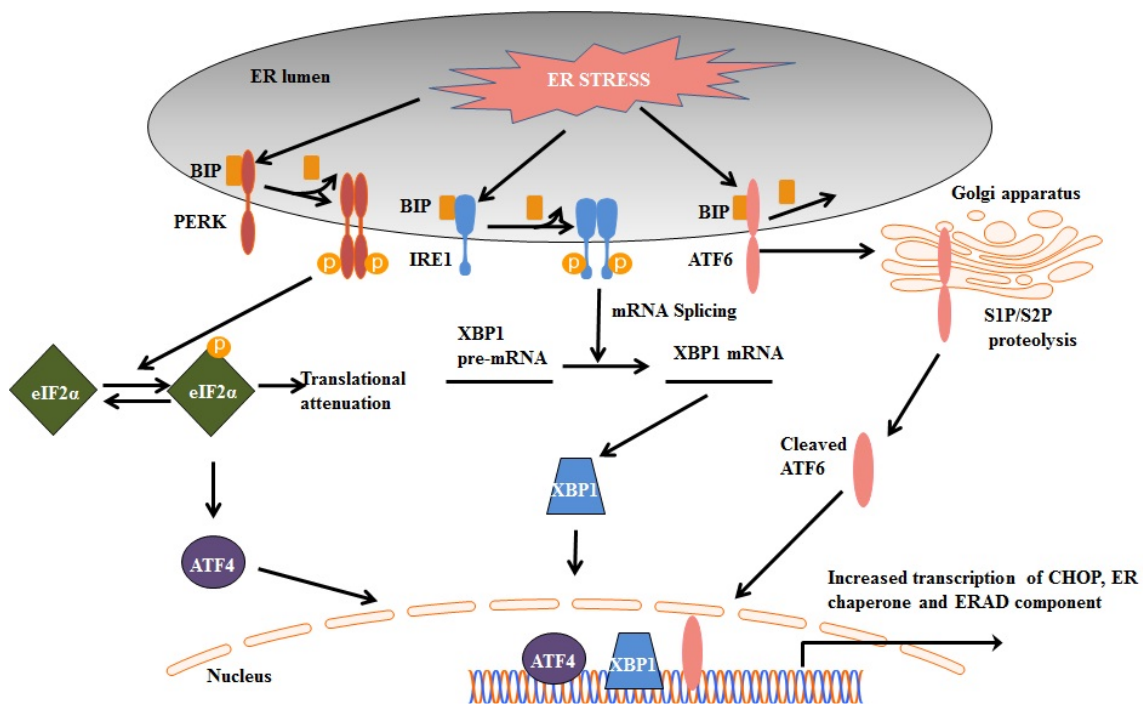


Fig. 1: Schematic Representation of ER Stress Signaling Pathway. Under ER stress, three branches of the UPR are activated. In PERK-eIF2 $\alpha$  branch, the activated PERKs form homodimers and phosphorylate eIF2 $\alpha$ , leading to inactivation of this protein and further the blockade of global translation. Another signaling event of the activation of the PERK signal pathway is an increase in translation of the transcription factor ATF4. Increase of ATF4 enhances the transcription of many downstream genes that plays an important role in amino acid biosynthesis, antioxidant stress responses, transport functions, and apoptosis. One of them is the transcription factor C/EBP homology (CHOP/GADD153), a classic ER stress marker (Verfaillie et al., 2010). In the IRE1-XBP1 branch, the activated IRE1 undergoes homodimerization, which results in trans autophosphorylation and production of mature XBP1 (spliced XBP1). Then the spliced XBP1 serves as a transcription factor that enters into the nucleus and increases transcription of genes needed for ER/Golgi biogenesis, lipid synthesis, and ER-associated protein degradation (ERAD). One of them is Bip, a known ER chaperone as well as a classic ER stress marker (Acosta-Alvear et al., 2007). In the ATF6 branch, the activated ATF6 translocates from ER lumen to the Golgi apparatus, where it is cleaved by a couple of proteases. After that, the spliced form of ATF6 acts as a transcription factor and enters into the nucleus to enhance transcription of genes such as GRP94, BiP, and calreticulin.

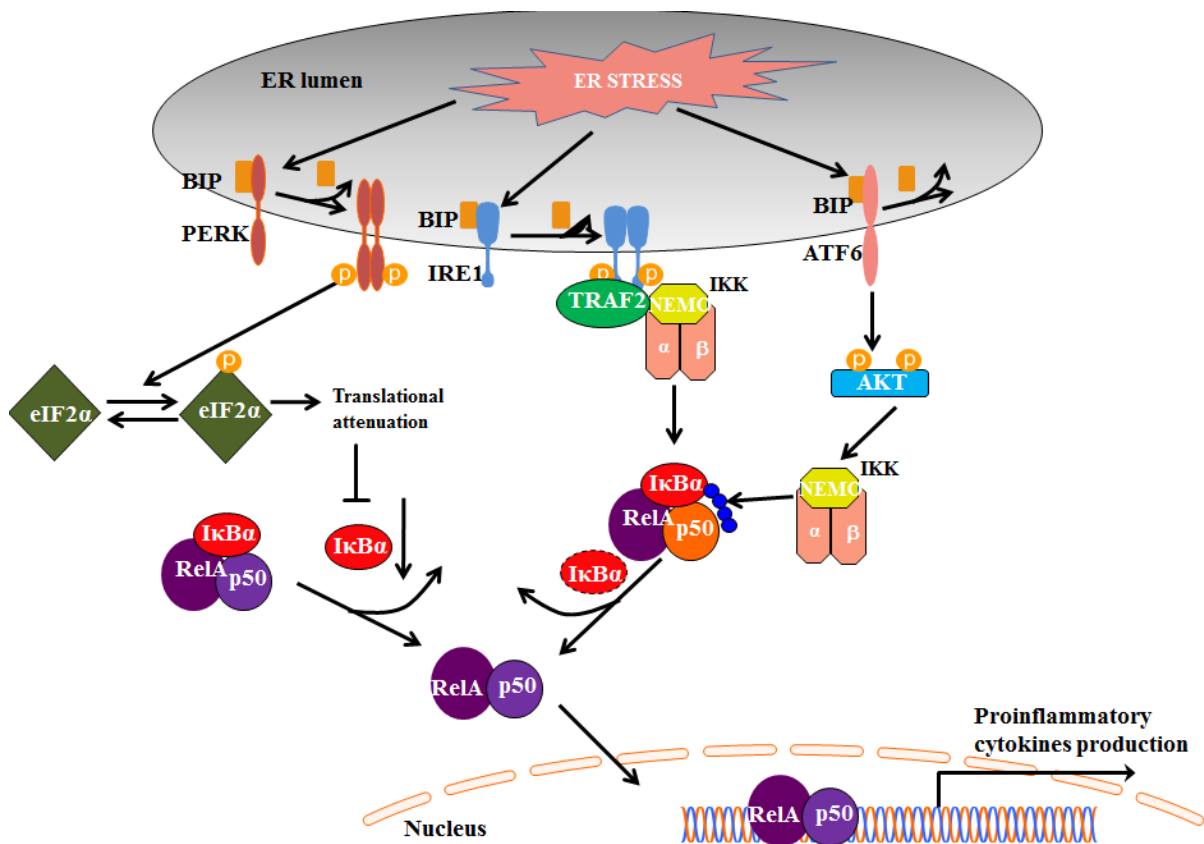
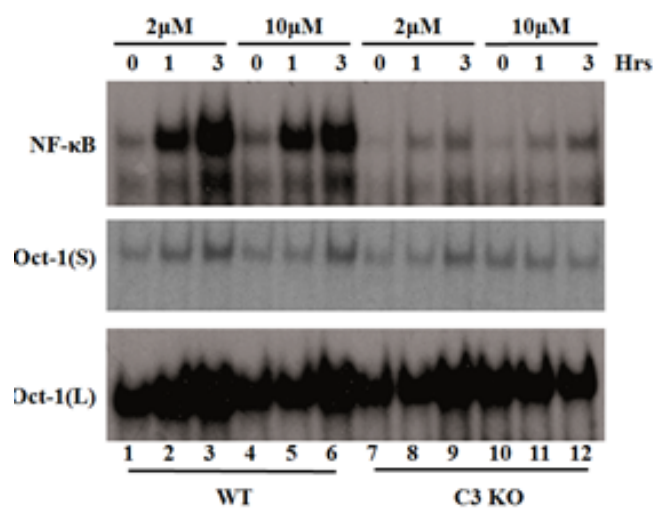
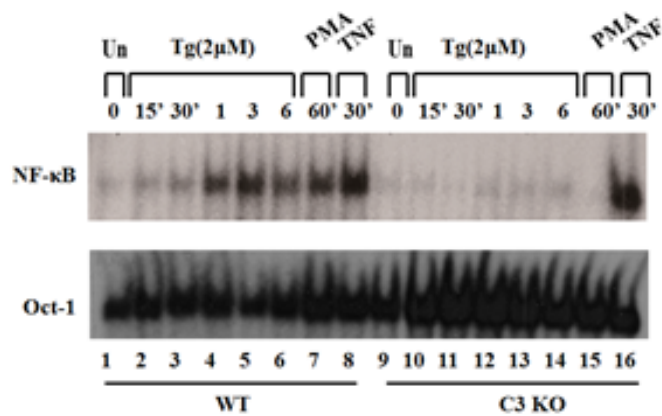


Fig. 2: Schematic Representation of IRE1 $\alpha$ -Mediated, ATF6-Mediated, and PERK-Mediated NF- $\kappa$ B Activation. Under ER stress, the activated PERK-eIF2 $\alpha$  arm causes translational arrest. This leads to the change in the ratio of NF- $\kappa$ B dimmers to I $\kappa$ B $\alpha$  in terms of protein level, since the half-life of I $\kappa$ B $\alpha$  protein was much shorter than NF- $\kappa$ B proteins in resting cells. Thus, there are no enough I $\kappa$ B $\alpha$  proteins to block NF- $\kappa$ B dimmers in the cytoplasm. Therefore, the “free” NF- $\kappa$ B dimmers enter into the nucleus and initiate the expression of downstream genes. In the IRE1 $\alpha$  branch, the activated IRE1 $\alpha$  can recruit and activate the IKK complex in a TRAF2-dependent manner. Then the activated IKK can further phosphorylate I $\kappa$ B $\alpha$  on Ser 32 and 34, and cause degradation of I $\kappa$ B $\alpha$  by proteasomes, and thereby freeing NF- $\kappa$ B dimmers. In the ATF6 branch, the activated ATF6 results in an activation of the AKT-IKK pathway.

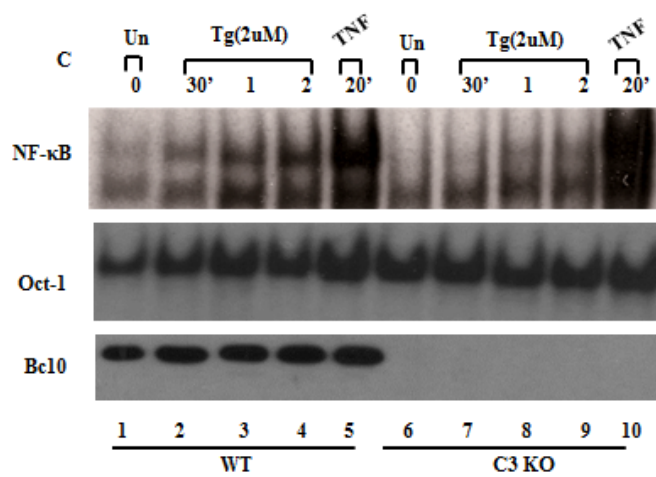
**A**



**B**



**C**



**D**

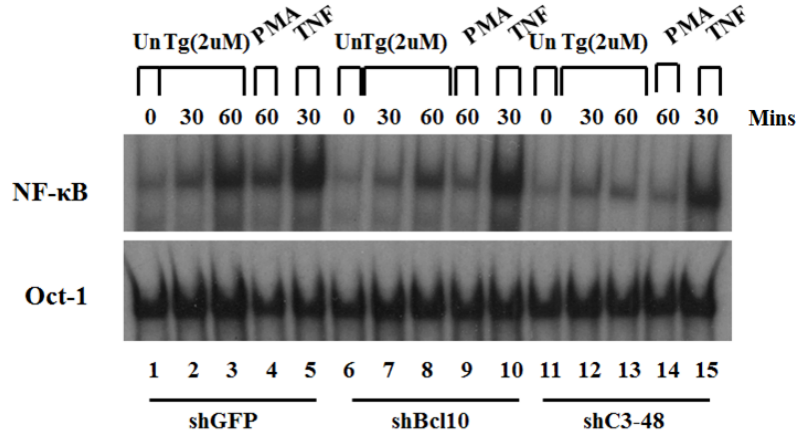


Fig. 3: Thapsigargin-Induced NF-κB Activation Is Mediated by CARMA3 and BCL10. At different time points, nuclear extracts were prepared from cells and then subjected to the electrophoretic mobility shift assay (EMSA) using <sup>32</sup>P-labeled NF-κB or OCT-1 probe. (A) WT and CARMA3-deficient (C3KO) MEF cells were either untreated or treated with different doses of thapsigargin. (B) WT and CARMA3-deficient(C3KO) MEF cells were either untreated or treated with 2 μM thapsigargin, 40 ng/ml PMA, and 10 ng/ml TNF for indicated time periods. (C) WT and BCL10-deficient(BCL10KO) MEF cells were either untreated or treated with 2 μM thapsigargin and 10 ng/ml TNF for indicated time periods. (D) Huh7 cells with the knockdown of GFP, CARMA3, and BCL10 were either untreated or treated with 2 μM thapsigargin, 40 ng/ml PMA and 10 ng/ml TNF.

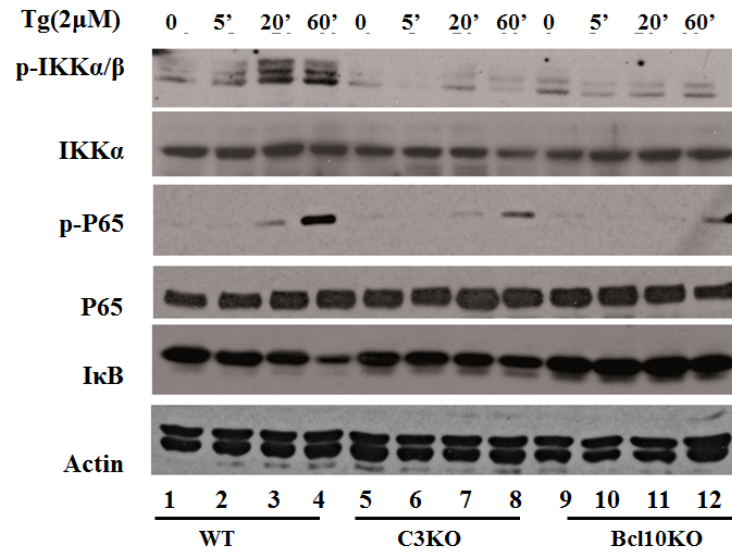
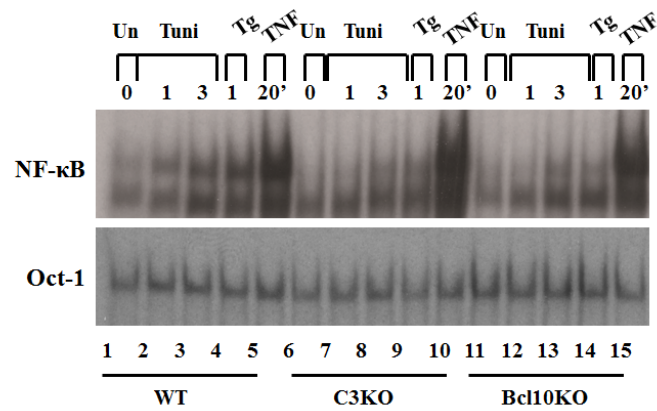


Fig. 4: CARMA3-Regulated IKK Complex Activation in Response to Thapsigargin Treatment Is Dependent on IKKα/β Phosphorylation. WT, BCL10-(BCL10KO) and CARMA3-deficient(CARMA3 KO) primary MEF cells were either untreated or treated with 2 μM thapsigargin for indicated time periods. Cell lysates were prepared and analyzed using immunoblotting using anti-phospho-IKKα/β (Ser<sup>176/180</sup>), anti-phospho-P65 (Ser<sup>536</sup>), anti-IKKα, anti-P65, anti-IκBα, and anti-β-actin antibodies.



A



B

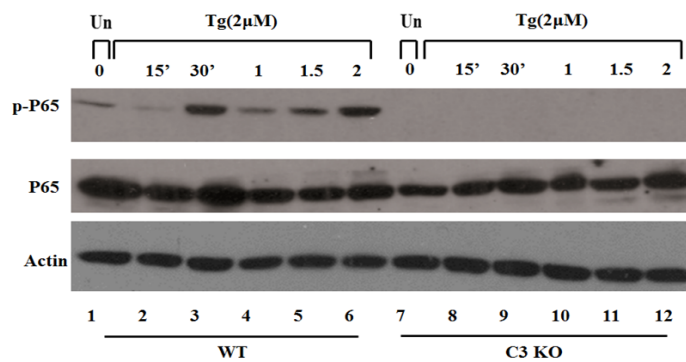


Fig. 5: CARMA3 and BCL10 Contribute to Tunicamycin-Induced NF-κB Activation in Primary MEF Cells. (A) WT, BCL10-(BCL10 KO) and CARMA3(C3 KO)-deficient primary MEF cells were either untreated or treated with 2 μM thapsigargin, 2ug/ml tunicamycin, and 10 ng/ml TNF. At different time points, nuclear extracts were prepared from cells and then subjected to the EMSA using <sup>32</sup>P-labeled NF-κB or OCT-1 probe. (B) Primary WT and CARMA3-deficient MEF cells were either untreated or treated with 2 μg/ml tunicamycin for indicated time periods. Cell lysates were prepared and analyzed using immunoblotting with anti-phospho-P65 (Ser<sup>536</sup>), anti-P65, and anti-β-actin antibodies.

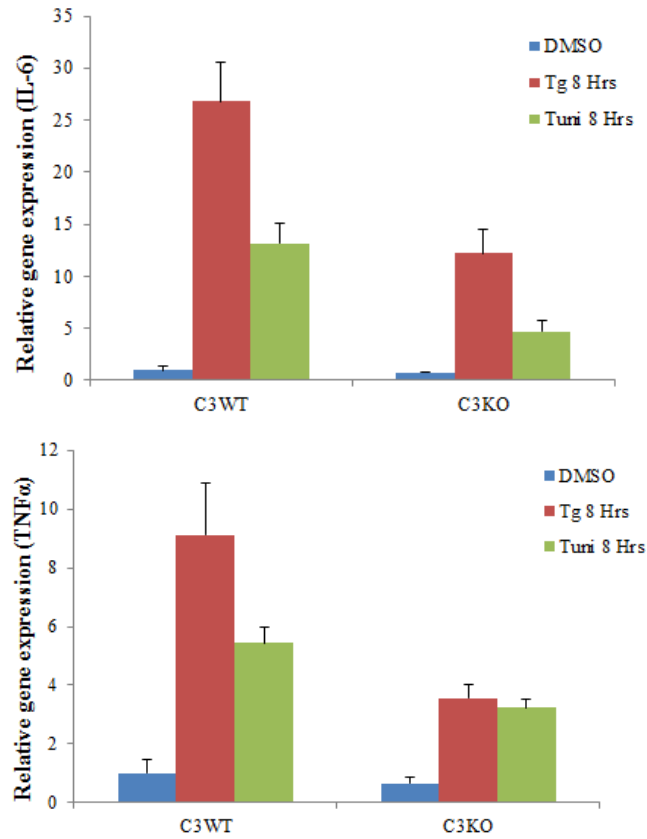
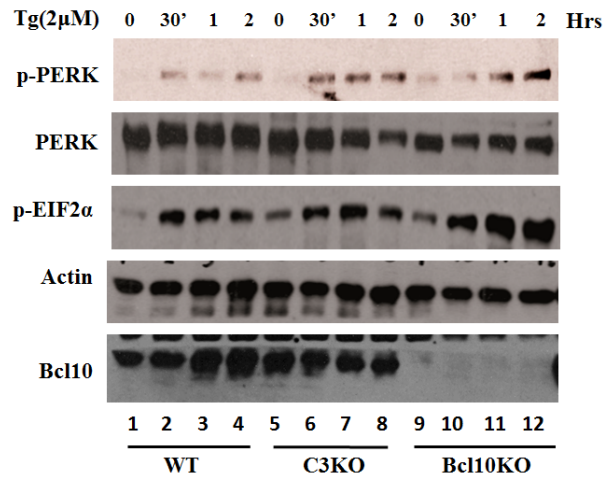


Fig. 6: CARMA3 Contributes to Production of ER Stress-Induced Proinflammatory Cytokines in MEF Cells. WT and CARMA3-deficient (C3 KO) MEF cells were either untreated or treated with 2  $\mu$ M thapsigargin and 2  $\mu$ g/ml tunicamycin for 8 hours. The mRNA level of mIL-6 and mTNF were detected using real-time quantitative PCR.

A



B

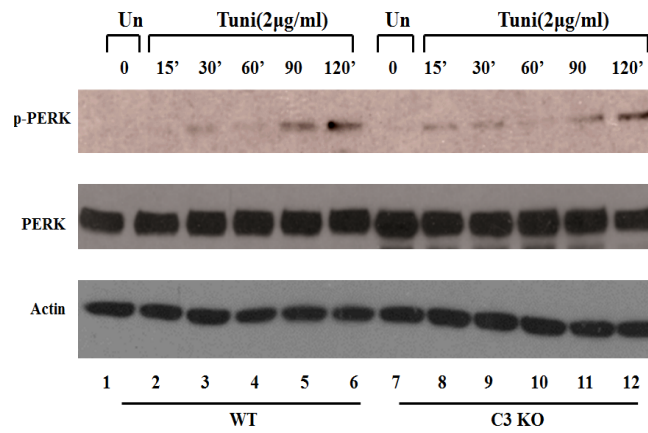


Fig. 7: Activation of the PERK Branch of the UPR Is Intact in CARMA3 Deficient Cells under ER Stress. (A) Primary WT, BCL10- and CARMA3-deficient (BCL10 KO; C3 KO) MEF cells were either untreated or treated with 2  $\mu$ M thapsigargin for indicated time spans. (B) Primary WT and CARMA3-deficient MEF cells were either untreated or treated with 2  $\mu$ g/ml tunicamycin. Cell lysates were prepared and analyzed using immunoblotting with anti-phospho-PERK (Thr980), anti-phospho-EIF2 $\alpha$  (Ser51), anti-PERK, and anti- $\beta$ -actin antibodies.

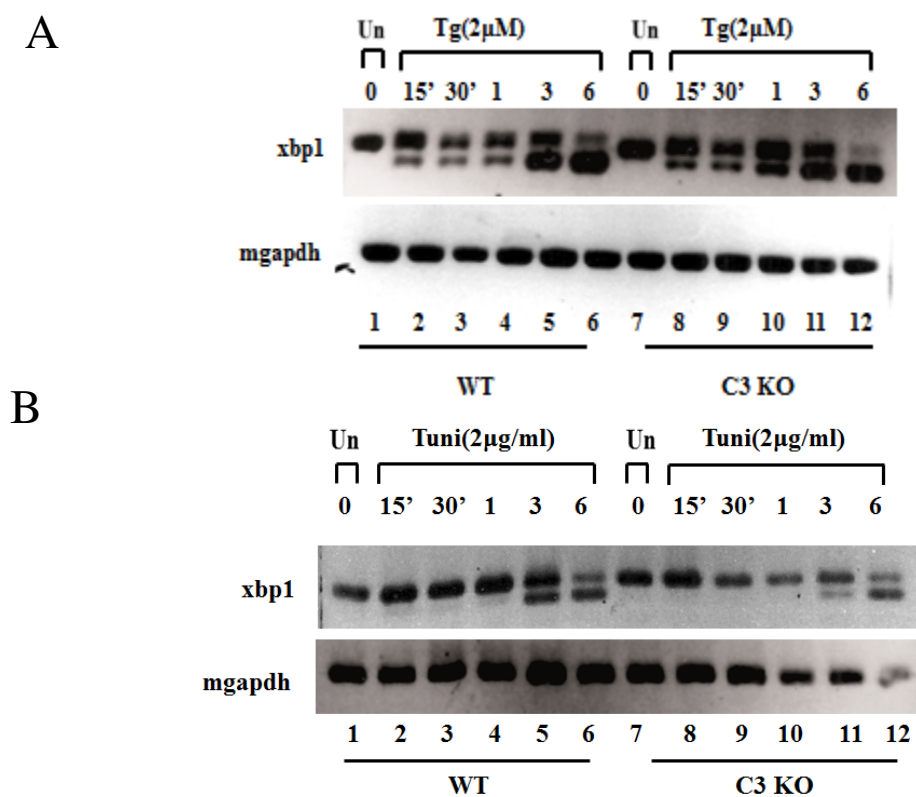


Fig. 8: Activation of the IRE1 $\alpha$  Branch of the UPR Is Intact in CARMA3-Deficient (C3 KO) Cells under ER Stress. Primary WT and CARMA3-deficient MEF cells were either untreated or treated with 2  $\mu$ M thapsigargin(A) and 2  $\mu$ g/ml tunicamycin(B), respectively. At different time points, xbp1 mRNA splicing products were analyzed using agarose gel electrophoresis.

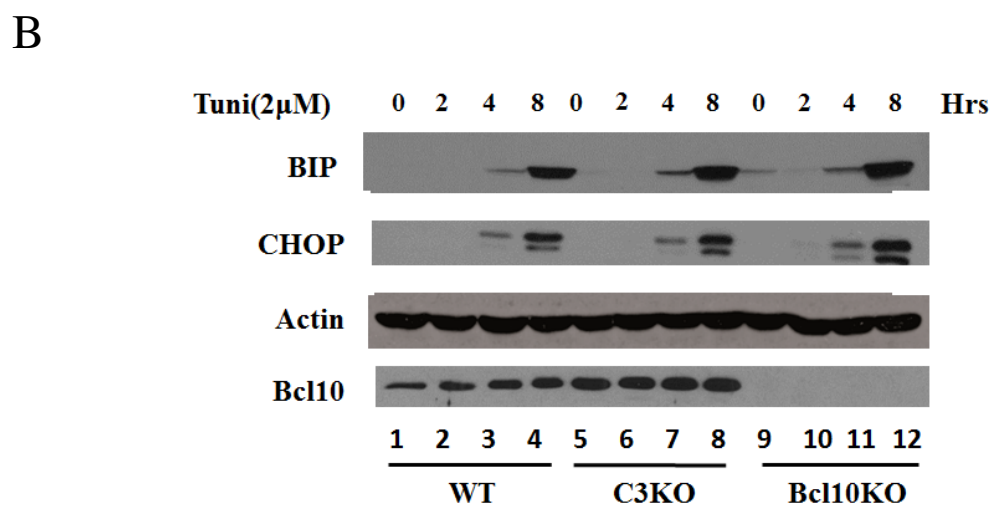
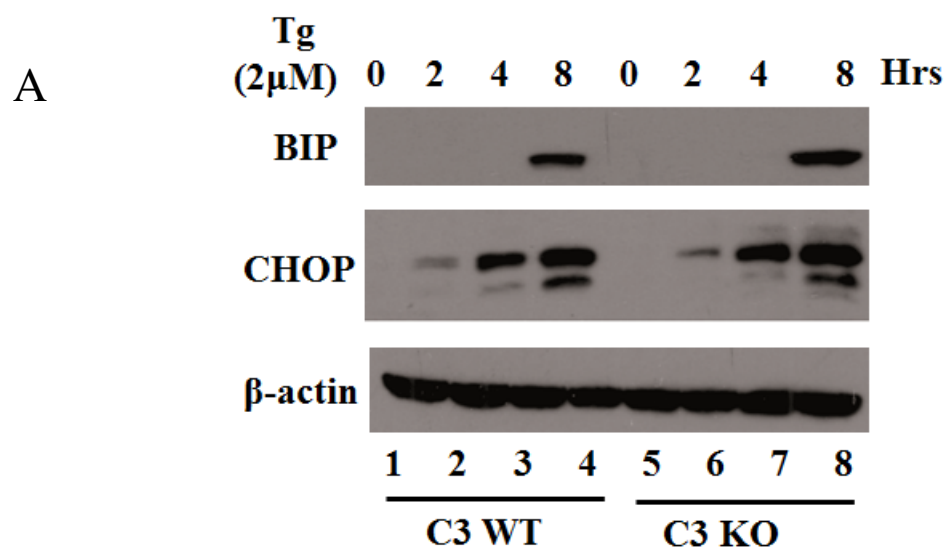


Fig. 9: ER Stress Signaling Pathway Is Intact In CARMA3-Deficient Cells Under ER Stress. Primary WT, BCL10-, and CARMA3-deficient MEF (BCL10 KO; CARMA3 KO) cells were either untreated or treated with 2 μM thapsigargin and 2 μg/ml tunicamycin, respectively. Cell lysates were prepared and analyzed using immunoblotting with anti-BIP, anti-CHOP, anti-BCL10, and anti-β-actin antibodies.

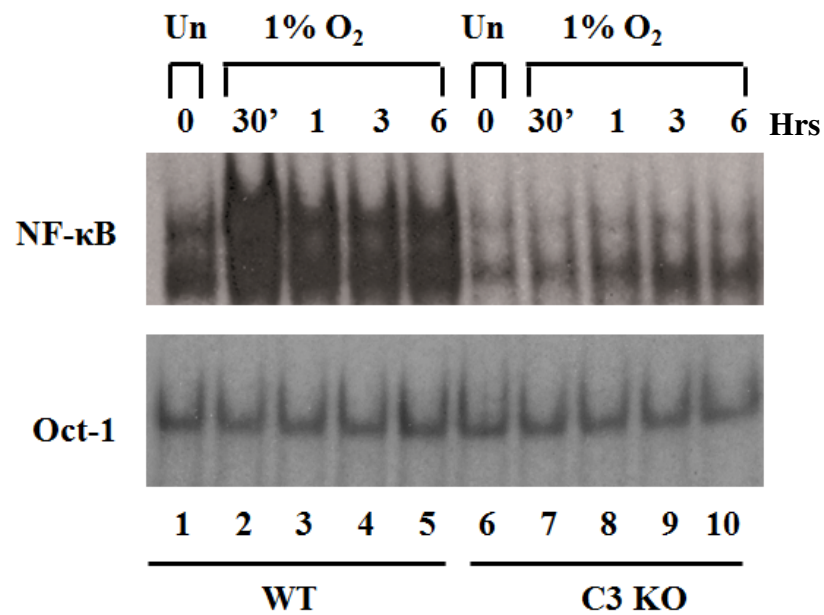


Fig. 10: CARMA3 Contributes to Hypoxia-Induced NF-κB. Primary WT and CARMA3-deficient MEF (C3 KO) cells were exposed to 1% O<sub>2</sub> for indicated time spans. Nuclear extracts were prepared from cells and then subjected to the EMSA using <sup>32</sup>P-labeled NF-κB or OCT-1 probe.

## CHAPTER 4: DISCUSSION

By using WT, BCL10- and CARMA3-deficient MEFs, we showed that thapsigargin- and tunicamycin-induced NF- $\kappa$ B activation were dependent on CARMA3 and BCL10. Consistently, we found that a physiological ER stress inducer, hypoxia, activated NF- $\kappa$ B in a CARMA3-dependent manner. Additionally, the UPR signaling pathway was found to be intact in both CARMA3- and BCL10-deficient cells under ER stress.

Recently, interest in ER stress-induced NF- $\kappa$ B activation has been increasing, since ER stress-induced inflammation was found to have an important role in many diseases, such as obesity, type II diabetes, cancers, and so on. What is more, NF- $\kappa$ B signaling pathway is a key regulator of inflammation (Cnop et al., 2012; Garg et al., 2012). More researches on ER stress-induced NF- $\kappa$ B will help investigators to develop better therapeutic methods for treating these diseases.

However, studies on activation of NF- $\kappa$ B under ER stress conditions are limited and somewhat controversial. Early studies provided three different mechanisms of how ER stress activates NF- $\kappa$ B. Specifically, the ER stress sensors IRE1, PERK, and ATF6 can activate NF- $\kappa$ B through activation of the IKK complex, attenuation of the I $\kappa$ B $\alpha$  translation, and the PI3K-Akt pathway, respectively (Hotamisligil, 2010). Theoretically, considering that activation of the three ER stress branches is independent of each other, defects of one of UPR branches or inhibitors blocking one pathway would only cause partial defects of NF- $\kappa$ B in cells under ER stress. However, that is not the case. Several studies showed completely defective NF- $\kappa$ B activation. One reasonable explanation is that different types of cells were

used in these studies. For example, Kaneko et al. (2003) used 293 cells and found that NF- $\kappa$ B activation under ER stress was transduced via IRE1 and TRAF2, whereas Jiang et al. (2003) used immortalized MEF cells and found that the PERK-EIF2 $\alpha$  arm of the UPR was required for activation of NF- $\kappa$ B in response to ER stress (Jiang et al., 2003; Kaneko et al., 2003; Yamazaki et al., 2009). Another reasonable explanation is that ER-stress induces NF- $\kappa$ B by integrating functions from UPR signaling pathways. The evidence from Tam et al.'s study supports it. They proposed that IKK basal level regulated by IRE1 and translation repression by PERK together determines cell sensitivity to ER stress-induced NF- $\kappa$ B activation. This may represent a novel mechanism of NF- $\kappa$ B activation. According to their model, ER stress works as an amplifier rather than inducer of NF- $\kappa$ B activity (Tam et al., 2012). Moreover, UV induces early NF- $\kappa$ B activation in a similar manner. These studies suggest the common mechanism of NF- $\kappa$ B activation shared by stresses, since many cellular stress conditions, such as viral infection, oxidative stress, heat shock, hypoxia, ribotoxic stress, and UV irradiation can inhibit protein synthesis to different extent (O'Dea et al., 2008). In our study, we used primary MEF cells to investigate the role of CARMA3 and BCL10 in ER stress-induced NF- $\kappa$ B activation, which was similar to Jiang et al. and Tam et al.'s studies. What is more, our data also showed that the basal level of NF- $\kappa$ B activity of CARMA3 deficient cells is decreased compared to that of WT. Therefore, it is logical to hypothesize that CARMA3 and BCL10 work in IRE1 signaling pathway to regulate basal IKK activity. However, our studies showed the inducible activation of IKK complex after 2 $\mu$ M thapsigargin stimulation, which is inconsistent with Tam et al.'s study (Fig. 4). It may be due to the different dose of thapsigargin used in two studies. In their paper, they used only



200nM thapsigargin, which was ten folds less than we used. Except for activation of UPR signaling pathways, thapsigargin can also raise cytosolic calcium. It is well established that intracellular calcium serves as second messengers to trigger different signal events to mediate NF- $\kappa$ B. For example, intracellular calcium can be absorbed by mitochondrial to produce reactive oxygen species (ROS)(Hom et al., 2010). It is well established that ROS can activate NF- $\kappa$ B through different mechanisms (Gloire et al., 2006). In addition, many calcium-dependent kinases can regulate NF- $\kappa$ B activity under different conditions. For instance, calcium/calmodulin-dependent protein kinase II phosphorylates CARMA1 on Ser109 and plays an important role in TCR signaling and CARMA1-induced NF- $\kappa$ B activation. Consistent with these, Fig.3A revealed that NF- $\kappa$ B signaling pathway was not completely defective in CARMA3-deficient cells after thapsigargin stimulation. We still detected an inducible activation of NF- $\kappa$ B in CARMA3-deficient cells at different time points. One reasonable explanation is the redundant ER stress-induced NF- $\kappa$ B signaling pathway. This may explain why P65 phosphorylation was partially defective in CARMA3-deficient cells following treatment. Therefore, more evidence is needed to figure out that the inducible IKK activation is due to the ER stress or other signal events. Another possibility is the secondary effect. Figure 4 showed that production of proinflammatory cytokine TNF was partially defective in CARMA3-deficient cells. It has been well established that TNF activates NF- $\kappa$ B in a CARMA3-independent manner (Grabiner et al., 2007). Additionally, TNF is a strong NF- $\kappa$ B inducer. Therefore, it is reasonable that we observe an inducible NF- $\kappa$ B activation in CARMA3-deficient MEF cells following thapsigargin treatment.

Recently, studies have shown that full activation of the IKK complex is controlled by two events, that is, phosphorylation of two kinases, IKK $\alpha$  and IKK $\beta$ , and the K63-linked ubiquitination of a regulatory subunit, NEMO/IKK $\gamma$  (Israel, 2010). Additionally, studies in our lab have indicated that CARMA3 works downstream of GPCR to mediate activation of the IKK complex by inducing NEMO ubiquitination but not IKK $\beta$  phosphorylation. CARMA3 is physically associated with NEMO/IKK $\gamma$  and may induce polyubiquitination of NEMO through an unknown binding pattern(s), likely by linking NEMO to TRAF6 (Grabiner et al., 2007). Inconsistent with what we found, we observed that IKK phosphorylation was defective in CARMA3-deficient MEF cells following thapsigargin treatment, which suggests a novel mechanism of how CARMA3 and BCL10 regulate IKK activation in response to ER stress. In ER stress signaling pathway, CARMA3 or BCL10 may recruit the IKK complex to IRE1, since the recruitment is important for IKK phosphorylation and activation (Bonnet et al., 2000; Gil et al., 2000). In other signaling pathways, CARMA3 or BCL10 just provides “a second signal” to activate the IKK complex (Blonska and Lin, 2011).

Present study has limitations. Firstly, our experiments relied only on primary MEF cells. Although the primary MEF cell is a powerful tool to study the mechanism, cell response to ER stress may depend on the type of cells and stress. Cell types that are most easily affected by ER stress include hepatocytes, pancreatic  $\beta$ -cells, adipocytes, and macrophage, since the trafficking of large amounts of proteins through the ER is required for maintaining normal cell functions (Kaneko et al., 2003). Therefore, any deregulation of ER

stress signaling pathway in these cells highly correlates with disease progression. For example, persistent and strong ER stress-induced inflammation in pancreatic  $\beta$  cells and hepatocytes contributes to insulin resistance and thus aids the progression of type II diabetes and obesity, respectively. Future studies will be focused on ER stress-induced inflammation and NF- $\kappa$ B activation in these cell types. Secondly, the study only used drugs to induce stress, even though thapsigargin and tunicamycin are the most commonly used drugs to study ER stress-related signaling pathways. Although we used hypoxia to study the role of CARMA3 in ER stress-induced NF- $\kappa$ B, the data is quite limited. Further studies are needed to investigate how CARMA3 works in hypoxia-induced NF- $\kappa$ B. Is the mechanism of thapsigargin- and tunicamycin-induced NF- $\kappa$ B similar to that of hypoxia-induced NF- $\kappa$ B?

Our study suggests a role of the CARMA3-BCL10 complex in ER stress-induced NF- $\kappa$ B activation, which may help to elucidate the mechanism of ER stress-induced NF- $\kappa$ B signaling and facilitates the development of the corresponding methods to release ER stress and maintain homeostasis in cells.

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