Ask1 gene deletion blocks maternal diabetes-induced endoplasmic reticulum stress in the developing embryo by disrupting the unfolded protein response signalosome

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ABSTRACT

Apoptosis signal-regulating kinase 1 (ASK1) is activated by various stresses. The link between ASK1 activation and endoplasmic reticulum (ER) stress, two causal events in diabetic embryopathy, has not been determined. We sought to investigate whether ASK1 is involved in the unfolded protein response (UPR) that leads to ER stress. Deleting Ask1 abrogated diabetes-induced UPR by suppressing phosphorylation of inositol-requiring enzyme 1 alpha (IRE1α) and double stranded RNA activated protein kinase (PKR)-like ER kinase (PERK), blocked the mitochondrial translocation of proapoptotic Bcl-2 members and ER stress. ASK1 participated in the IRE1α signalosome, and removing ASK1 abrogated the proapoptotic kinase activity of IRE1α. Ask1 deletion suppressed diabetes-induced IRE1α endoribonuclease activities, which led to X-box binding protein 1 mRNA cleavage, an ER stress marker; decreased expression of microRNAs; and increased expression of a miR-17 target, thioredoxin-interacting protein (Txnip), a thioredoxin binding protein, which enhanced ASK1 activation by disrupting the thioredoxin-ASK1 complexes. ASK1 is essential for the assembly and function of the IRE1α signalosome, which forms a positive feedback loop with ASK1 through Txnip. ASK1 knockdown in C17.2 neural stem cells diminished high glucose- or tunicamycin-induced IRE1α activation, which further supports our hypothesis that ASK1 plays a causal role in diabetes-induced ER stress and apoptosis.
Pregnancy with pre-existing maternal diabetes significantly increases the risk of neural tube defects (NTDs) in the offspring (1; 2). Maternal diabetes disrupts the balance between intracellular reactive oxygen species and endogenous antioxidant capacities in neurulation stage embryos, leading to NTD formation (3-6). Oxidative stress induced-apoptosis (7-10) and gene dysregulation (7; 11; 12) are the central mechanisms underlying diabetic embryopathy. ASK1, an oxidative stress responsive kinase, is activated by maternal diabetes, and ASK1 plays a causal role in the induction of apoptosis in the neuroepithelium and NTD formation (10). However, the downstream intermediates that transmit the proapoptotic response of ASK1 in diabetic embryopathy are unknown. It has been shown that ASK1 is a key component of the unfolded protein response (UPR) signalosome that leads to ER stress (13; 14), a proapoptotic response in diabetic embryopathy (15; 16).

The ER, a specialized intracellular organelle, is responsible for correct folding and three-dimensional structures of secretory and transmembrane proteins. Protein folding is carried out by a group of ER-resident molecular chaperone proteins including binding immunoglobulin protein (BiP) and calnexin. Accumulation of unfolded proteins caused by physiological or pathological cues perturbs ER function, resulting in UPR activation (17). Three ER transmembrane proteins, IRE1α, PERK (Protein kinase RNA-like endoplasmic reticulum kinase), and activated transcription factor 6 alpha, act as sensors of the UPR signaling (17). Transient UPR signaling is beneficial because it provides an adaptive response to resolve ER stress through inhibiting protein translation, increasing ER chaperone expression, and facilitating unfolded protein degradation (17).
However, prolonged UPR signaling transforms this adaptive response into a severe form of ER stress, which induces apoptosis (18). This severe form of ER stress has been observed in diabetic embryopathy (15).

ER stress plays an important role in the pathogenesis of diabetes by triggering beta-cell dysfunction and apoptosis (19). ER stress contributes to the etiology of diabetic retinopathy through induction of retinal vascular and neuronal cell apoptosis (20). Among the three UPR sensors, IRE1α signaling appears to be the most important. It is responsible for the entire UPR in yeast (21). In addition, IRE1α plays a critical role in mediating the downstream effect of UPR because it possesses both protein kinase and endoribonuclease (RNase) activities (14; 22). IRE1α cleaves X-box binding protein 1 (XBP1) mRNA, resulting in a spliced XBP1 mRNA which is translated into an active transcription factor (21), which primarily stimulates expression of genes that encode ER chaperones in the UPR pathway (23). Apoptosis caused by prolonged UPR activation is mediated by IRE1α (24; 25). IRE1α interacts with TNF receptor-associated factor 2 (TRAF2) and ASK1, forming a proapoptotic signalosome, the IRE1α-TRAF2-ASK1 complex (13; 14). This complex triggers the mitochondrial apoptotic pathway by activating the proapoptotic Bcl-2 family members Bax and Bak (24). In addition, IRE1α increases the expression of other proapoptotic factors, including caspase 2 and Txnip, through cleavage of a group of microRNAs (miRs) (25).

ER stress activates ASK1 through the formation of the IRE1α-TRAF2-ASK1 complex (14). However, it is unknown whether ASK1 activation itself is sufficient to cause ER stress through promoting the assembly of IRE1α signalosome. Both ASK1 and ER stress are involved in maternal diabetes in vivo- or high glucose in vitro-induced
apoptosis in the developing neural tube leading to NTD formation (10; 15). Because the downstream kinases of ASK1, c-Jun-N-terminal kinase 1/2 (JNK1/2), are responsible for maternal diabetes-induced ER stress in the developing embryo (15), we hypothesize that maternal diabetes-induced ASK1 activation causes ER stress in the developing embryo. ASK1 is a proapoptotic kinase, whose activation leads to the activation of JNK1/2, p38MAPK or both (26). JNK1/2 play central roles in the progression of insulin resistance and beta-cell dysfunction and are potential therapeutic targets for diabetes (27). JNK1/2 mediates the pro-apoptotic effect of ER stress leading to retinal neuron apoptosis in diabetic retinopathy (28). We have previously demonstrated that targeted deletion of the Ask1 gene or its downstream kinases, Jnk1 or Jnk2, blocks maternal diabetes-induced apoptosis and NTD formation (15). In this study, we employed an Ask1 gene deletion mouse model to explore the relationship between ASK1 and the IRE1α-ER stress pathway in diabetic embryopathy, and assessed the role of ASK1 in tunicamycin- or high glucose-induced UPR activation.
Research Design and Methods

Animals and reagents

Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Ask1−/− mice on C57BL/6J background were provided by Dr. Hidenori Ichijo at the University of Tokyo. Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in sterile 0.1 M citrate buffer (pH 4.5). Sustained-release insulin pellets (Linplant) were purchased from Linshin, Canada. The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Mouse models of diabetic embryopathy

Our mouse model of diabetic embryopathy has been described previously (10; 15). Briefly, ten-week old WT and Ask1−/− female mice were intravenously injected daily with 75 mg/kg STZ over two days to induce diabetes. Diabetes was defined as a 12 hours fasting blood glucose level of ≥ 14 mM. Insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. Male and female mice were paired at 3:00 P.M., and pregnancy was identified by the presence of the vaginal plug next morning, and noon of that day was designated as day 0.5 (E0.5). On E5.5, insulin pellets were removed to make sure the developing embryos would be exposed to a hyperglycemic environment during neurulation (E8-10.5). WT and Ask1−/− mice were treated with vehicle injection and sham operations as nondiabetic controls.. On E8.75 (at 6:00 P.M.), mice were euthanized and Conceptuses were dissected out of the uteri, embryos with the yolk sacs were removed from the decidua and then yolk sacs were
removed from the embryos. The embryos were used for biochemical and molecular analysis.

**Cell culture, transfection and tunicamycin treatment**

C17.2 mouse neural stem cells, originally obtained from ECACC (European Collection of Cell Culture), were maintained in DMEM (5mM glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. The C17.2 cells are newborn mouse cerebellar progenitor cells transformed with retroviral v-myc (29). ASK1-siRNA (sc-29749) and control siRNA-A (sc-37007) were obtained from Santa Cruz Biotechnology (Dallas, TX). Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY) was used according to the manufacturer’s protocol for transfection of siRNA into the cells. After seeded for 24 hours, cells were transfected with siRNA and cultured in 1% FBS for 84 hours. Tunicamycin (T7765) (Sigma) or DMSO (final concentration is 0.04% DMSO volume/total medium volume) as control were added to growth medium for another 6 hours and then cells were harvested. To determine the effect of the ER stress inhibitor, 4-PBA(30), on high glucose-induced ER stress, cells were cultured in high glucose DMEM (25mM) media with 2mM 4-Phenylbutyric acid (4-PBA) for 48 hours.

**Immunoprecipitation (IP) and immunoblotting**

IP and immunoblotting were performed as previously described (10; 31). For IP, 300 µg protein from 5-6 embryos pooled per litter per immunoprecipitation, protease inhibitor cocktail (Sigma), lysis buffer (Cell Signaling Technology), Protein A Magnetic bead
slurry (New England BioLabs), 1 µg rabbit anti-ASK1 antibody (Santa Cruz Biotech) and mouse anti-thioredoxin (Abcam) were used. Mitochondria were isolated from embryos using the Pierce mitochondria isolation kit. For immunoblotting, equal amounts of protein (30 or 50 µg) from one whole embryo per dam were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immunobilon-P membranes (Millipore, Billerica, MA). 2 µg Precision Plus Protein Standards (Bio-Rad) were loaded into one lane of the gel. Membranes were incubated in 5% nonfat milk for 45 minutes and then were incubated for 18 hours at 4 °C with the following primary antibodies in 5% nonfat milk: rabbit anti-BiP, rabbit anti-calnexin, rabbit anti-CHOP (C/EBP homologous protein), rabbit anti-phospho-(p-)PERK, rabbit anti-(p-)IRE1α, rabbit anti-(p-)eIF2α, rabbit anti-(p-)ASK1, rabbit anti-ASK1, rabbit anti-Txnip, anti-caspase 2 and rabbit anti-caspase 3. Detailed antibody information is listed in Supplementary Table 1. Following primary antibody incubation, membranes were exposed to goat anti-rabbit or anti-mouse secondary antibodies. To ensure that equivalent amounts of protein were loaded among samples; membranes were stripped and probed with a mouse antibody against β-actin with dilution of 1:5000 (Abcam) and prohibitin (1:2000) (Calbiochem). Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). Three embryos from three different dams were used for immunoblotting. Embryos in replicate lanes in immunoblotting were from different litters and replicate IPs were performed using separate litters.

RNA extraction and Real-time PCR (RT-PCR)
Total RNA was isolated from embryos using an RNeasy Mini Kit (Qiagen) and reverse transcribed by using 2 µg RNA from one embryo and the high-capacity cDNA archive kit (Applied Biosystem). 1 µg microRNA was extracted by using the mirVana miRNA isolation kit (Ambion, Life Technologies) and reverse transcribed using NCode VILO miRNA cDNA synthesis kit (Invitrogen, Life Technologies). RT-PCR using 1/100 RT products for ER stress chaperone genes (calnexin, BiP, eIF2α, PDIA, GRP94, IRE1α), ASK1, β-actin (normalization control for mRNA), mmu-mir-17, mmu-mir-34a, mmu-mir-96, mmu-mir-125b and mmu-mir-U6 (normalization control for microRNA), were performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific, Rockford, IL). Primer sequences for miR and mRNA detection are listed in Supplementary Table 2. RT-PCR and subsequent calculations were performed by a StepOnePlus™ Real-Time PCR System (Applied Biosystem). Five embryos from five different dams were studied for RT-PCR

Detection of XBP1 mRNA splicing

The mRNA of XBP1 was extracted from E8.75 embryos and reverse-transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). The PCR primers for XBP1 were: forward, 5’-GAACCAGGAGTTAAGAACACG-3’ and reverse, 5’-AGGCAACAGTGTCAGAGTCC-3’. If no XBP1 mRNA splicing occurred, a 205-bp band was produced. When XBP1 splicing occurred, a 205 bp band and a 179 bp main band were produced.

TUNEL Assay
The TUNEL assay was performed as previously described by using the In Situ Cell Death Detection Kit (Millipore) (15). Cells were seeded on 8-well Nunc Lab-Tek Chamber Slide system (Sigma). After transfection and tunicamycin treatment, cells were fixed with 4% paraformaldehyde in PBS and incubated with TUNEL reagent, counterstained with DAPI, and mounted with aqueous mounting medium (Sigma). TUNEL-positive cells in each well were counted. The percentage of apoptotic cells was calculated as the number of TUNEL-positive (apoptotic) cells, divided by the total number of cells in a microscopic field and multiplied by 100 from three separate experiments.

Statistics

Data are presented as means ± SE. One-way ANOVA was performed using SigmaStat 3.5 software, a Tukey test was used to estimate the significance. Statistical significance was accepted when $p<0.05$. 

Diabetes
Results

*Ask1 deletion abrogates the activation of two UPR sensors*

Nondiabetic (ND) and diabetic (DM) WT or *Ask1*–/– female mice were bred with matched genotype ND males to produce WT and *Ask1*–/– embryos for studying the role of ASK1 in the UPR in diabetic embryopathy. Levels of maternal blood glucose and insulin, litter sizes in both WT and ASK1 KO diabetic mice are similar (Fig 1a). Protein levels of phosphorylated (p)-IRE1α, p-PERK, and p-eIF2α, a direct downstream effector of p-PERK, were significantly increased by maternal diabetes (Fig 1b, c, d). Deleting *Ask1* gene abolished the increase of p-PERK, p-IRE1α, and p-eIF2α by maternal diabetes (Fig. 1b, c, d).

*Ask1 gene deletion blocks severe ER stress in embryos exposed to diabetes*

Maternal diabetes induces severe ER stress in the developing embryos, which causes apoptosis in the neural tube and NTD formation (15). Because ASK1 is a key component of the IRE1α signalosome (14), whose activation causes ER stress (25), we tested whether *Ask1* gene deletion blocked ER stress. We analyzed protein levels of ER stress markers including BiP, calnexin, and CHOP. Consistent with our previous findings (15; 16), protein levels of BiP, calnexin, and CHOP were significantly higher in WT embryos from diabetic dams than those in WT embryos of nondiabetic dams (Fig.2a, b, c). Levels of these three ER stress markers in *Ask1*–/– embryos of nondiabetic and diabetic dams were significantly lower when compared with those in WT embryos of diabetic dams, and were similar to levels of ER stress markers seen in WT embryos of nondiabetic dams (Fig.2a, b, c).
Elevated ER chaperone gene expression also is indicative of ER stress. Maternal diabetes significantly increased the expression of five ER chaperone genes: BiP, calnexin, eIF2α, PDIA3 and GRP94 (Fig. 2d). The levels of expression of these five ER chaperone genes were significantly increased in WT embryos of diabetic dams and were reduced in Ask1−/− embryos of diabetic dams to levels that did not differ from WT or Ask1−/− embryos of nondiabetic dams (Fig. 2d). The mRNA level of IRE1α did not differ among embryos from any of the groups (Fig. 2d). This result indicates that the total expression level of IRE1α did not change under diabetic conditions, but that the active form of phosphorylated-IRE1α was significantly increased by maternal diabetes and suppressed by Ask1−/− condition (Fig.1a). eIF2α mRNA was increased (Fig. 2d) whereas eIF2α protein was not affected by diabetes (Fig. 1c). Increased eIF2α phosphorylation (Fig. 1c) may explain this discrepancy, because eIF2α phosphorylation is associated with its rapid degradation (32).

Deleting Ask1 abolishes the proapoptotic kinase activity of IRE1α

To test whether maternal diabetes induces the proapoptotic complex, IRE1α-ASK1-TRAF2, we performed an IP assay using an ASK1 antibody. The abundance of p-IRE1α, TRAF2 and p-ASK1 in ASK1 immunoprecipitates was significantly higher in embryos of diabetic dams than in embryos of nondiabetic dams (Fig. 3a), indicating that maternal diabetes enhances the formation of the IRE1α-ASK1-TRAF2 complex. An anti-p-ASK1 IP further confirmed the assembly of the IRE1α-ASK1-TRAF2 complex under diabetic conditions and in the absence of ASK1, this complex was not formed (Fig. 3a).
The IRE1α-ASK1-TRAF2 complex mediates the proapoptotic response of IRE1α by inducing the translocation of proapoptotic Bcl-2 family members to the mitochondria (24). To determine whether ASK1 is responsible for the translocation of Bax and Bak, two key proapoptotic Bcl-2 members, mitochondria were isolated from embryos and the amount of mitochondrial Bax and Bak was assessed. Bax and Bak translocated and accumulated in the mitochondria of embryos exposed to maternal diabetes, but not in WT embryos of nondiabetic dams, or in Ask1−/− embryos of nondiabetic or diabetic dams (Fig. 3b). Bax and Bak levels in whole embryonic lysates did not differ (Fig. 3b). These results suggest that Ask1 gene deletion abrogated the proapoptotic response emanating from the IRE1α-ASK1-TRAF2 complex.

**Ask1 deletion diminishes the RNase activity of IRE1α**

Upon activation, p-IRE1α possesses RNase activities and splices XBP1 mRNA into a potent transcriptional activator to induce UPR responsive genes and aggravate ER stress. To determine whether ASK1 has any influence on maternal diabetes-induced XBP1 mRNA splicing, XBP1 mRNA splicing was measured by a RT-PCR. WT embryos exposed to maternal diabetes exhibited robust splicing manifested with two bands at 205 bp (very weak) and 179 bp of the XBP1 PCR products, whereas WT embryos from nondiabetic dams showed no XBP1 splicing, and had only one band (205 bp) of the PCR products (Fig. 4a). In Ask1−/− embryos exposed to maternal diabetes, XBP1 mRNA splicing was not detected (Fig. 4a).

The IRE1α RNase activity also causes rapid decay of a group of microRNAs (miRs) including miR-17, miR-34a, miR-96 and miR-125b, and thus relieves the
degradation and/or translation repression of these miR-targeted mRNAs (25). Maternal diabetes significantly suppressed the levels of miR-17, miR-34a and miR-96 but did not affect the expression of miR-125 (Fig. 4b). Under diabetic conditions, Ask1 gene deletion restored the levels of miR-17, miR-34a and miR-96 to the levels in embryos from nondiabetic dams (Fig. 4b).

**ASK1 differentially regulates two miR target genes**

Txnip is a target gene of miR-17, while caspase 2 is a target gene of miR-17, miR-34a and miR-96 (25; 33). We measured the mRNA and protein levels of Txnip and caspase 2. Consistent with the reduction of miR-17 by diabetes, both mRNA and protein levels of Txnip were significantly increased in WT embryos of diabetic dams (Fig. 5a, b). Under diabetic conditions, Ask1 gene deletion reduced the mRNA and protein levels of Txnip to those in embryos of nondiabetic dams (Fig. 5a, b).

Total mRNA and protein levels of caspase 2 did not show any significant difference in embryos from nondiabetic and diabetic dams (Fig. 5c, d). Levels of cleaved caspase 2 were significantly increased by maternal diabetes (Fig. 5d). Ask1 deletion abolished maternal diabetes-induced caspase 2 cleavage (Fig. 5d). These results suggest that Txnip expression is regulated by ASK1 and IRE1α associated miRs, whereas caspase 2 expression is not regulated by maternal diabetes, ASK1 and IRE1α. However, caspase 2 is activated through ASK1 by maternal diabetes.

**The miR target gene, Txnip, sequesters thioredoxin from ASK1 under diabetic conditions**
Increased Txnip binds to the active cysteine residue of thioredoxin (34) and suppresses the inhibitory effect of thioredoxin on the kinase activity or phosphorylation of ASK1 (35). Indeed, the interaction of thioredoxin and Txnip was significantly increased by maternal diabetes, while the interaction of thioredoxin and ASK1 was significantly decreased by maternal diabetes (Fig. 6), suggesting that the increase in Txnip expression by maternal diabetes removes the inhibitory effect of thioredoxin on ASK1 activation. Consistent with this notion, and as previously reported (10), p-ASK1 levels were increased by maternal diabetes (Fig. 6)

**ASK1 knockdown attenuates tunicamycin-induced UPR and severe ER stress in vitro**

Our results demonstrated that ASK1 is essential for diabetes-induced UPR signalosome and signalosome in the developing embryo. To further confirm the causal relationship between ASK1 and UPR/ER stress, we examined the effect of ASK1 on tunicamycin-induced UPR and severe ER stress in C17.2 neural stem cell line, which was used as a complementary *in vitro* approach. To test the effect of ASK1 on proteins involved in the UPR in the cell culture model, ASK1 mRNA was transiently inhibited with siRNA. ASK1 siRNA treatment reduced ASK1 mRNA and protein levels to 75% and 80% of those in controls, respectively (Fig. 7a). ASK1 Knockdown abrogated tunicamycin-induced IRE1α phosphorylation (Fig. 7b), ER chaperones (BiP and CHOP) expression (Fig. 7b), down-regulation of miR expression (Fig. 7c), stimulation of Txnip expression (Fig. 7d), XBP1 splicing (Fig. 7e) and ER stress-induced caspase cleavage and apoptosis (Fig. 7f, g).
ASK1 knockdown and 4-Phenylbutyric acid (4-PBA) treatment block high glucose-induced UPR and miRs/Txnp expression, respectively.

ASK1 Knockdown inhibited high glucose-induced PERK, eIF2α, and IRE1α phosphorylation (Fig. 8a) and CHOP expression (Fig. 8a). 4-PBA treatment restored high-glucose-induced down-regulation of miR expression (Fig. 8b) and up-regulation of Txnip expression (Fig. 8c).
Discussion

A schematic diagram of the proposed pathway is shown in Fig. 8d. The IRE1α UPR pathway has two main functions: pro-apoptotic kinase signaling and RNase activity (36). Unfolded protein accumulation in the ER induces IRE1α oligomerization, which juxtaposes the kinase domain of IRE1α and consequently triggers kinase activation via autophosphorylation (37-39). IRE1α kinase activation directly leads to its RNase activity (21). In the absence of ASK1, both IRE1α kinase and RNase activities are diminished, suggesting that ASK1 is essential for both activities.

We postulate two possible mechanisms for ASK1 involvement in IRE1α activation. First, ASK1 alone or with the whole IRE1α-TRAF2-ASK1 complex acts as a scaffold protein for IRE1α aggregation and is essential for IRE1α activation (40). Indeed, a recent study identified another cytosolic factor that is required for IRE1α aggregation and optimal activation (41). Second, although initial or transient IRE1α activation via autophosphorylation is triggered by the accumulation of unfolded/misfolded proteins in the ER lumen, prolonged IRE1α activation may require an additional mechanism (42). While transient IRE1α activation resolves homeostatic imbalance in the ER lumen, prolonged IRE1α activation results in ER stress (42). ASK1 may directly phosphorylate IRE1α at sites other than the IRE1α autophosphorylation site. The IRE1α RNase activity is the result of its phosphorylation (22). Because ASK1 deletion abolishes IRE1α phosphorylation, ASK1 deletion suppresses the IRE1α RNase activity by restoring a group of miRs expression, which is undergoing rapid decay due to IRE1α activation.
The TRAF2-ASK1-JNK1/2 pathway is the first mechanism known to mediate the proapoptotic effect of IRE1α. Because JNK1/2 induces translocation of proapoptotic Bcl-2 family members to the mitochondria, the activation of the TRAF2-ASK1-JNK1/2 pathway leads to apoptosis by, presumably, activating the mitochondrial pathway (43). Indeed, the proapoptotic Bcl-2 members, Bax and Bak, are translocated and accumulated in the mitochondria in embryos exposed to diabetes. In this pathway, ASK1 plays a central role because deleting Ask1 abrogates Bax and Bak mitochondrial translocation.

Recently, the RNase activity of IRE1α was linked to proapoptotic effects by cleaving microRNAs, which repress the expression of pro-apoptotic factors (25). Due to reduced expression of miR-17, miR-17 target gene, Txnip, increases. Txnip has emerged as a new proapoptotic factor (44; 45). It appears that the IRE1α RNase activity mediates the proapoptotic response through down-regulation of a distinct set of miRs and up-regulation of miR target genes including Txnip. Increased Txnip, a thioredoxin binding protein, sequesters thioredoxin from the ASK1-thioredoxin complexes, and thus activates ASK1. Maternal diabetes-induced reactive oxygen species oxidizes the endogenous ASK1 inhibitor, thioredoxin (Trx), which dissociates from ASK1 and thereby causing ASK1 activation (10). The transcription factor, FoxO3a, relays the pro-apoptotic signal of ASK1 to the nucleus by inducing the expression of an apoptotic gene, TRADD (10). In a separate study, we demonstrated that TRADD is an active component of the IRE1α signalosome through its binding to TRAF2. The association between TRADD and TRAF2 is known in the cell death receptor pathway (46). IRE1α signalosome-increased Txnip contributes to ASK1 activation. Thus, the IRE1α signalosome and the ASK1-FoxO3a-Caspase 8 pathway are interlinked in diabetic embryopathy.
Our findings in the tunicamycin study support the critical role of ASK1 in ER stress induced by diabetes. This is important because different factors which induce ER stress contribute to the etiology of different human diseases. Tunicamycin induces severe ER stress and apoptosis by disrupting protein folding in the ER through inhibition of N-linked glycosylation (47; 48). siRNA knockdown of ASK1 abrogates tunicamycin-induced IRE1α phosphorylation, ER chaperone expression and ER stress-induced apoptosis. Thus, the causal role of ASK1 in persistent UPR activation and ER stress may also be applicable to pathophysiological conditions other than diabetic embryopathy.

Apoptosis is directly involved in the induction of diabetic embryopathy (7-10; 49). The IRE1α signalosome is the main mediator of ER stress-induced apoptosis (13; 24; 25), suggesting that IRE1α plays a crucial role in the induction of diabetic embryopathy. IRE1α knockout mice are embryonic lethal, but mice with conditional IRE1α deletion only in the embryo proper do not exhibit any adverse defects (50). Future studies using conditional deletion of Ire1α will be able to uncover the role of IRE1α in diabetes-induced neural tube defects.

There are some shortcomings to our experimental strategies. For one, we used a homozygous breeding strategy (WT or Ask1−/−) instead of breeding nondiabetic or diabetic heterozygous Ask1+/− females to nondiabetic Ask1+/− males. Therefore, the embryos differ from each other, not only by their genotypes and a diabetic or nondiabetic maternal environment, but also by maternal genotype and any effects the maternal genotype would have on experimental results. However, a heterozygous breeding scheme would be much more laborious, as embryos would need to be genotyped individually before assays. Additionally, whole embryos were used for assays, and so, we do not know if ASK1
signaling and ER stress are limited to embryonic neuroepithelium. Another shortcoming is that, while the results of the C17.2 cell line are supportive of the embryo results, the C17.2 cell line may not truly reflect the cell biology of the embryonic neuroepithelium. This cell line was derived from neonatal cerebellum, which is developmentally more differentiated than neuroepithelium of the neurulating embryo, and it has been immortalized by retroviral transduction with v-myc (29). Nevertheless, high glucose culture of C17.2 cells mimicked hyperglycemia in vivo by inducing UPR signaling, ER stress, and apoptosis in this cell line. The C17.2 cell line provided a feasible model for our ASK1 knockdown experiments, which supported the causal role of ASK1 in the induction of IREα signalosome and ER stress.

In summary, ASK1 is essential for both diabetes and tunicamycin or high glucose in vitro-induced ER stress. Our study reveals the causal role of ASK1 in persistent UPR activation, consequent ER stress and apoptosis (Fig. 8d).
Author Contributions

F. W, Y. W. and H. G. researched data. P. Y. conceived the project and designed the experiments. P. Y. and F. W. wrote the manuscript and E. A. R., S.F., R. G. B. and G. A. participated in data analyses and reviewed the manuscript. All authors have approved the final version of the paper.

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**Figure Legends**

Figure 1. *Ask1* deletion diminishes maternal diabetes-induced UPR sensor activation. a, blood glucose levels at E8.75, insulin levels at E8.75 and litter size (n = 5 means 5 dams per group). b, levels of p-IRE1α. c, levels of p-PERK, and d, levels of p-eIF2α. In b, c, d, representative lanes were from 1 embryo with bar graphs showing means +/- SEM of 3 lanes of 3 embryos (n = 3), each from a separate dam. b, c, d were separate gels of different embryonic samples. Within each panel, the membrane was probed first for the phosphorylated protein and sequentially stripped for probing total protein, ASK1 and β-actin. This is the case for all figures. * indicate significant differences (p<0.05) compared to the other three groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type. a, b, & c were separate gels, and were reprobed for separate proteins in each panel.

Figure 2. *Ask1* deletion blocked maternal diabetes-induced ER stress markers and ER chaperone genes expression. Protein levels of BiP (a), calnexin (b) and CHOP (c). In d, mRNA levels of ER chaperone genes (n =5). In a, b and c, experiments were repeated three times using embryos of three different dams (n = 3) per group. * significant differences compared to the other three groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type. a, b, & c were separate gels, and were reprobed for separate proteins in each panel.

Figure 3. Maternal diabetes enhances the formation of the IRE1α-ASK1-TRAF2 complex, and *Ask1* deletion blocks mitochondrial translocation of Bax and Bak. a, Representative images of immunoprecipitation (IP) using an ASK1 antibody and an anti-
p-ASK1 antibody. In the bar graphs, levels of p-IRE1α, TRAF2 and p-ASK1 were assessed in ASK1 or p-ASK1 immunoprecipitates and were normalized by respective levels in 10% input. Normal rabbit IgG was used as controls. b, Protein levels of Bax and Bak in mitochondrial extracts were normalized by the mitochondrial marker, prohibitin. Total Bax and Bak levels in whole tissue lysates were also determined. In a and b, embryos from one litter per group were used per IP run or mitochondria extraction and three litters (n = 3) per group were used. * significant differences compared to the other groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type. a, and b were separate gels, and were reprobed for separate proteins in each panel.

Figure 4. ASK1 is required for the RNase activity of IRE1α. a, XBP1 mRNA splicing was detected in E8.75 embryos by reverse transcription and subsequent PCR. n = 2 mean two embryos from separate dams per group. Although the levels of cleaved XBP1 PCR product could be quantified, quantitative data might not be necessary because XBP1 cleavage was only detected in WT embryos of the DM group. b, Levels of miR-17, miR-125b, miR-96 and miR-34a. Experiments were repeated three times using embryos of three different dams (n = 3) per group. * indicate significant differences compared to the other three groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type.

Figure 5. Ask1 deletion blocks maternal diabetes-induced Txnip up-regulation and Caspase 2 cleavage. Levels of mRNA and protein levels of Txnip (a and b) and levels of mRNA and protein levels of Caspase 2 (c and d). Cleaved Caspase 2 was indicated in d.
Experiments were repeated three times using embryos of three different dams (n = 3) per group. * indicate significant differences compared to the other three groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type. b and d were separate gels, and were reprobed for separate proteins in each panel.

Figure 6. Maternal diabetes alters ASK1 and Txnip association with Trx in diabetic embryopathy. Representative images of immunoprecipitation (IP) using an ASK1 antibody. In the bar graphs, levels of p-IRE1α, TRAF2 and p-ASK1 were assessed in ASK1 immunoprecipitates and were normalized by respective levels in 10% input. Normal rabbit IgG was used as controls. Embryos from one litter per group were used per IP run and three litters (n = 3) per group were used. * significant differences compared to the other group. ND: nondiabetic; DM: diabetic mellitus; Trx: Thioredoxin.

Figure 7. ASK1 knockdown blocks tunicamycin-induced IRE1α activation, ER stress and apoptosis in C17.2 neural stem cells. a, mRNA and protein levels of ASK1. b, protein levels of p-IRE1α, IRE1α, BiP, CHOP, ASK1 and β-actin. c, miR expression. d, Txnip mRNA levels. e, XBP1 mRNA splicing. f, levels of total and cleaved caspase 2 and 3. In a, b, c, e, f, experiments were repeated three times (n = 3). g, representative images of the TUNEL assay. Apoptotic cells were labeled as Red and all cell nuclei were stained as Blue. Experiments were repeated three times (n = 3) and the quantification of the data were shown in the bar graph. Tm: tunicamycin. * indicate significant difference compared to other groups (p<0.01).
Figure 8. ASK1 knockdown suppresses high glucose-induced IRE1α activation and ER stress, 4-PBA blocks high glucose-increased miR and Txnip expression in C17.2 neural stem cells. a, protein levels of p-IRE1α, p-PERK, p-eIF2α, CHOP, ASK1 and β-actin. b, levels of miRs. c, levels of Txnip mRNA and protein. In a, b, c, experiments were repeated three times (n = 3). * indicate significant difference compared to other groups.

In d, a schematic diagram shows that ASK1 plays essential role in the assembly of the IRE1α signalosome which leads to prolonged UPR, severe ER stress and apoptosis. Maternal diabetes activates ASK1 by oxidizing its inhibitor, Trx and simultaneously removes the ER luminal inhibition of IRE1α. Maternal diabetes induces the formation of IRE1α-TRAF2-ASK1 complexes, which is indispensable for the proapoptotic kinase and the RNase activities of IRE1α. Ask1 deletion disrupts the IRE1α-TRAF2-ASK1 complexes and thus blocks the proapoptotic kinase and the RNase activities of IRE1α. Disruption of the IRE1α signalosome also inhibits PERK activation. Maternal diabetes increases Txnip expression, a downstream effector of the IRE1α RNase activity. Txnip sequesters thioredoxin from ASK1 and thus enhances ASK1 activation.
Figure 1

(a) Blood glucose level (mM)

- **WT**
- **ASK1-/-**

ND: 0, 10, 20, 30

DM: * *

(b) Insulin level (ng/ml)

- **WT**
- **ASK1-/-**

ND: 0.0, 0.4, 0.8, 1.2

DM: * *

(c) Embryos number per litter in 5 dams

- **WT**
- **ASK1-/-**

ND: 0, 2, 4, 6, 8

DM: * *
Figure 1

**b**

<table>
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<tr>
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<th>WT</th>
<th>ASK1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
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- **p-IRE1α**: 110KD
- **IRE1α**: 110KD
- **ASK1**: 155KD
- **β-actin**: 42KD

Graph showing the ratio of **P-IRE1α/IRE1α** in WT and ASK1<sup>−/−</sup> conditions under ND and DM conditions. The graph indicates a significant increase in the ratio of P-IRE1α/IRE1α in ASK1<sup>−/−</sup> compared to WT in the DM condition (marked with an asterisk).
Figure 1

c

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p-PERK/PERK

WT: 
ASK1−/−:

p-PERK/PERK

WT: 
ASK1−/−:

p-eIF2α/eIF2α

WT: 
ASK1−/−:

p-eIF2α/eIF2α

WT: 
ASK1−/−:
Figure 2

(a) Western blot analysis of BiP, ASK1, and β-actin in WT and ASK1−/− mice under ND and DM conditions. BiP levels are highlighted at 78KD, ASK1 at 155KD, and β-actin at 42KD.

(b) Western blot analysis of calnexin and β-actin in WT and ASK1−/− mice under ND and DM conditions. Calnexin levels are highlighted at 90KD and 155KD, β-actin at 42KD.
Figure 2

CHOP

ASK1

β-actin

<table>
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<tr>
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<th>WT</th>
<th>ASK1⁻/⁻</th>
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<td>β-actin</td>
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</table>

WT      ASK1⁻/⁻   WT      ASK1⁻/⁻
ND      DM       ND      DM

![Graph showing CHOP/β-actin expression levels for WT and ASK1⁻/⁻ in ND and DM conditions.]

* indicates statistical significance.
Figure 2

**Calnexin mRNA/β-actin mRNA**

- **WT**
- **ASK1**

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<tr>
<td><strong>ASK1</strong></td>
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**BIP mRNA/β-actin mRNA**

- **WT**
- **ASK1**

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<td><strong>ASK1</strong></td>
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**eIF2α mRNA/β-actin mRNA**

- **WT**
- **ASK1**

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<td><strong>ASK1</strong></td>
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**BiP mRNA/β-actin mRNA**

- **WT**
- **ASK1**

<table>
<thead>
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**PDIA mRNA/β-actin mRNA**

- **WT**
- **ASK1**

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**GRP94 mRNA/β-actin mRNA**

- **WT**
- **ASK1**

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**IRE1α mRNA/β-actin mRNA**

- **WT**
- **ASK1**

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<td><strong>ASK1</strong></td>
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</table>
Figure 3

Diabetes

IP: IgG ASK1

WB: p-IRE1α

IRE1α

TRAF2

p-ASK1

ASK1

10% input

110KD 155KD

TRA2F2

53KD

p-ASK1

155KD

155KD

110KD

53KD

155KD

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM

ND ND DM
Figure 3

**Bax**

Prohibitin

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**Bak**

Prohibitin

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**Bax/prohibitin**

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**Bak/prohibitin**

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<tr>
<td>DM</td>
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**Total embryo lysates**

**Bak**

**Bax**

**β-actin**

<table>
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**Bak/β-actin**

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<tr>
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**Bax/β-actin**

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Figure 4

XBP1 splicing

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<tr>
<td></td>
<td>ASK1-KO ASK1-KO</td>
<td>ASK1-KO ASK1-KO</td>
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</table>

- **XBP1**
  - 205 bp
  - 179 bp

- **GAPDH**
Figure 4

(b) miR-17

miR-125b

miR-96

miR-34a

Diabetes

Page 40 of 55
Figure 5

(a) 

**Txnip mRNA**

- WT
- ASK1−/−

<table>
<thead>
<tr>
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<th>ASK1−/−</th>
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<tbody>
<tr>
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<tr>
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</table>

(b) 

**Western Blot Analysis**

- **Txnip**
  - 44KD
- **ASK1**
  - 155KD
- **β-actin**
  - 42KD

<table>
<thead>
<tr>
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<th>WT</th>
<th>ASK1−/−</th>
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<tbody>
<tr>
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<tr>
<td>DM</td>
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</tbody>
</table>

**Diabetes**
Figure 5

(c) Caspase 2 mRNA

(d) Cleaved Caspase 2

WT     ASK1

Cleaved Caspase2

WT     ASK1

ND     DM

WT     ASK1

ND     DM

Caspase2

β-actin

30KD

155KD

42KD

Caspase 2 mRNA

Caspase2

Cleaved Caspase2

ASK1

β-actin

30KD

155KD

42KD

Caspase 2 mRNA

Caspase2

Cleaved Caspase2

ASK1

β-actin

30KD

155KD

42KD

Caspase 2 mRNA

Caspase2

Cleaved Caspase2

ASK1

β-actin

30KD

155KD

42KD

Caspase 2 mRNA

Caspase2

Cleaved Caspase2

ASK1

β-actin

30KD

155KD

42KD

Caspase 2 mRNA

Caspase2

Cleaved Caspase2

ASK1

β-actin

30KD

155KD

42KD
Figure 7

a.

**ASK1 mRNA**

<table>
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<tr>
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<th>0</th>
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<td>ASK1 mRNA/β-actin mRNA</td>
<td>[bar chart]</td>
<td>[bar chart]</td>
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siRNA (84 hrs) 0 Control ASK1 (30pmol)

**ASK1 protein**

- 155KD
- 42KD

**β-actin**

- 155KD
- 42KD
Figure 7

**b**

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Diabetes
Figure 7

**d**

**Txnip mRNA**

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**e**

**XBP1**

**GAPDH**

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**205 bp band/GAPDH**

**179 bp band/GAPDH**
Figure 7

Cleaved caspase 2/β-actin

0.0
0.5
1.0

* *

Control
ASK1 (30pmol)
siRNA (84hrs)

Tm (µg/ml)
0 4 0 4 0 4

Cleaved caspase 3/β-actin

0.0
0.4
0.8
1.0

* *

Control
ASK1 (30pmol)
siRNA (84hrs)

Tm (µg/ml)
0 4 0 4 0 4

ASK1

42KD
155KD

Caspase2

Cleaved caspase 2
30KD

Caspase3

Cleaved caspase 3
17KD

β-actin

42KD
Figure 7

Diabetes

Blank Control

Control siRNA

ASK1 siRNA

4µg/ml Tm

Control siRNA +4µg/ml Tm

ASK1 siRNA +4µg/ml Tm

% TUNEL positive cells

0 10 20 30 40 50

siRNA (84hrs) 0 Control ASK1 (30pmol)

Tm (µg/ml) 0 4 0 4 0 4
Figure 8

(a) Western blot analysis showing phosphorylation levels of IRE1α, PERK, eIF2α, CHOP, ASK1, and β-actin under different conditions.

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(b) Quantitative analysis of phosphorylation levels:

- p-IRE1α/IRE1α
- p-PERK/PERK
- p-eIF2α/eIF2α
- CHOP/β-actin

* indicates statistical significance.
Diabetes

Figure 8

b

Glucose level (mM)
2mM 4-PBA
5  25  25
-  -  +

Mmu-mir-17/mmu-mir-U6
0.0
0.4
0.8
miR-17

Glucose level (mM)
2mM 4-PBA
5  25  25
-  -  +

Mmu-mir-125b/mmu-mir-U6
0.0
0.4
0.8
miR-125b

Glucose level (mM)
2mM 4-PBA
5  25  25
-  -  +

Mmu-mir-96/mmu-mir-U6
0.0
0.4
0.8
miR-96

Glucose level (mM)
2mM 4-PBA
5  25  25
-  -  +

Mmu-mir-34a/mmu-mir-U6
0.0
0.4
0.8
miR-34a
Figure 8

**c**

<table>
<thead>
<tr>
<th>Glucose level (mM)</th>
<th>5</th>
<th>25</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mM 4-PBA</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Txnip mRNA**

- 5 mM glucose: 
  - 25 mM 4-PBA: lower Txnip mRNA expression

**β-actin**

- 5 mM glucose: 
  - 25 mM 4-PBA: higher β-actin expression

Diabetes

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Figure 8 d

Maternal diabetes

Oxidative stress

Thioredoxin

oxidization

ASK1

TRAF2

IRE1α

Foxo3a

Endoribonuclease activity

miRs degradation

XBP1 splicing

Txnip

ER stress

p-PERK

Endoribonuclease activity

Caspase activation

Apoptosis
**SUPPLEMENTAL DATA**

**Supplementary Table 1.** The dilutions and sources of antibodies in immunoblotting.

<table>
<thead>
<tr>
<th>Antibodies name</th>
<th>Catalog number</th>
<th>Antibody sources</th>
<th>Dilution method</th>
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</thead>
<tbody>
<tr>
<td>p-IRE1α</td>
<td>Ab48187</td>
<td>Abcam</td>
<td>1:1000</td>
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<tr>
<td>IRE1α</td>
<td>3294s</td>
<td>Cell Signaling technology</td>
<td>1:2000</td>
</tr>
<tr>
<td>p-PERK</td>
<td>3179s</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<td>PERK</td>
<td>3192s</td>
<td>Cell Signaling technology</td>
<td>1:2000</td>
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<tr>
<td>p-eIF2α</td>
<td>3597s</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<tr>
<td>eIF2α</td>
<td>2103s</td>
<td>Cell Signaling technology</td>
<td>1:2000</td>
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<tr>
<td>BiP</td>
<td>3177</td>
<td>Cell Signaling technology</td>
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<tr>
<td>Calnexin</td>
<td>2679s</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>CHOP</td>
<td>2895s</td>
<td>Cell Signaling technology</td>
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<tr>
<td>p-ASK1</td>
<td>3765</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<tr>
<td>ASK1</td>
<td>sc7931</td>
<td>Santa Cruz Biotechnology</td>
<td>1:2000</td>
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<tr>
<td>Txnip</td>
<td>SAB2102616-50UG</td>
<td>Sigma-Aldrich</td>
<td>1:1000</td>
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<tr>
<td>Thioredoxin (Trx)</td>
<td>ab16845-100</td>
<td>Abcam</td>
<td>1:1000</td>
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<tr>
<td>TRAF2</td>
<td>4724s</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<tr>
<td>Bax</td>
<td>2172s</td>
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<tr>
<td>Bak</td>
<td>12105s</td>
<td>Cell Signaling technology</td>
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<tr>
<td>Prohibitin</td>
<td>CP34</td>
<td>Calbiochem</td>
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<td>Caspase 3</td>
<td>ab1899</td>
<td>EMD Millipore</td>
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<tr>
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<td>NB110-55655</td>
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<tr>
<td>β-actin</td>
<td>Ab8224</td>
<td>Abcam</td>
<td>1:5000</td>
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**Supplementary Table 2.** The sequences of primers used in RT-PCR.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sources</th>
<th>Primer sequences</th>
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</thead>
<tbody>
<tr>
<td>GRP94F</td>
<td>Primerbank ID: 6755863a1</td>
<td>TCGTCAGAGCTGATGATGAAGT</td>
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<tr>
<td>GRP94R</td>
<td></td>
<td>GCGTTTAACCACCATCCACTGAAT</td>
</tr>
<tr>
<td>CalnexinF</td>
<td>Primerbank ID: 6671664a1</td>
<td>ATGGAAGGGAAGTGTTGTTACTGT</td>
</tr>
<tr>
<td>CalnexinR</td>
<td></td>
<td>GCTTTGATAGTGACCTTTTGGAG</td>
</tr>
<tr>
<td>eIF2αF</td>
<td>Primerbank ID: 6857781a1</td>
<td>AGTCCCTGCTGAATCTCTCCT</td>
</tr>
<tr>
<td>eIF2αR</td>
<td></td>
<td>TCCCAAGGCAGACAGATATACC</td>
</tr>
<tr>
<td>PDIA3F</td>
<td>Primerbank ID: 6679687a1</td>
<td>CGCCTCCGATGTGTGGGA</td>
</tr>
<tr>
<td>PDIA3R</td>
<td></td>
<td>CAGTGCAATCCACCTTTGCTAA</td>
</tr>
<tr>
<td>IRE1αF</td>
<td>Primerbank ID: 13249351a1</td>
<td>ACACGGACCACCTATCTCA</td>
</tr>
<tr>
<td>IRE1αR</td>
<td></td>
<td>CTCAGGATAATGGTAGCCATGTC</td>
</tr>
<tr>
<td>BiPF</td>
<td>Primerbank ID: 31981722a1</td>
<td>ACTTGGGGACCACCTATTCC</td>
</tr>
<tr>
<td>BiPF</td>
<td></td>
<td>ATCGCCAATCAGACGCCTCC</td>
</tr>
<tr>
<td>TxnipF</td>
<td>Primerbank ID: 118131130c1</td>
<td>GGCGGGACGGTTAGTATGG</td>
</tr>
<tr>
<td>TxnipR</td>
<td></td>
<td>AGCGCAAGTAGCCATGTC</td>
</tr>
<tr>
<td>Caspase2F</td>
<td>Primerbank ID: 6680848a1</td>
<td>GGAGCAGGATTTTGGCAGTG</td>
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<tr>
<td>Caspase2R</td>
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<td>GCCTGGGGTCTCTCTTTT</td>
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<td>ASK1F</td>
<td>Primerbank ID: 171846248c3</td>
<td>TGACACACACCAAACAGGT</td>
</tr>
<tr>
<td>ASK1R</td>
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<td>CGAGGGGTAAGCAGATGG</td>
</tr>
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<td>Mmu-mir-17</td>
<td>Own design</td>
<td>CAAAGTGCTTACAGTGAGG</td>
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<tr>
<td>Mmu-mir-96</td>
<td>Own design</td>
<td>GGACTAGCACATTTTTGTCA</td>
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<tr>
<td>Mmu-mir34a</td>
<td>Own design</td>
<td>GGCGATGCATTAGCTGGG</td>
</tr>
<tr>
<td>Mmu-mir125b</td>
<td>Own design</td>
<td>CCTGAGACCCCTAAGT</td>
</tr>
</tbody>
</table>

F: forward; R: reverse. Mmu: murine.