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Melatonin reduces endoplasmic reticulum stress and corneal dystrophy-associated TGFBIp through activation of endoplasmic reticulum-associated protein degradation

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Abstract

Endoplasmic reticulum (ER) stress is emerging as a factor for the pathogenesis of granular corneal dystrophy type 2 (GCD2). This study was designed to investigate the molecular mechanisms underlying the protective effects of melatonin on ER stress in GCD2. Our results showed that GCD2 corneal fibroblasts were more susceptible to ER stress-induced death than were wild-type cells. Melatonin significantly inhibited GCD2 corneal cell death, caspase-3 activation, and poly (ADP-ribose) polymerase 1 cleavage caused by the ER stress inducer, tunicamycin. Under ER stress, melatonin significantly suppressed the induction of immunoglobulin heavy-chain-binding protein (BiP) and activation of inositol-requiring enzyme 1α (IRE1α), and their downstream target, alternative

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splicing of X box-binding protein 1. Notably, the reduction of BiP and IRE1α by melatonin was suppressed by the ubiquitin proteasome inhibitor, MG132, but not by the autophagy inhibitor, bafilomycin A1, indicating involvement of the ER-associated protein degradation (ERAD) system. Melatonin treatment reduced the levels of transforming growth factor-β-induced protein (TGFBIp) significantly, and this reduction was suppressed by MG132. We also found reduced mRNA expression of the ERAD system components *HRD1* and *SEL1L*, and a reduced level of SEL1L protein in GCD2 cells. Interestingly, melatonin treatments enhanced SEL1L levels and suppressed the inhibition of SEL1L N-glycosylation caused by tunicamycin. In conclusion, this study provides new insights into the mechanisms by which melatonin confers its protective actions during ER stress. The results also indicate that melatonin might have potential as a therapeutic agent for ER stress-related diseases including GCD2.

1. INTRODUCTION

The endoplasmic reticulum (ER) is responsible for the quality control of newly synthesized proteins through protein processing, glycosylation, and disulfide bond formation.[1] Various physiological and pathological conditions can induce perturbations of ER homeostasis such as the accumulation of unfolded or misfolded proteins. To reduce this stress, the ER triggers a signaling cascade called the unfolded protein response (UPR).[2,3] This adaptive stress response leads to translation attenuation, elimination of misfolded proteins via ER-associated degradation (ERAD), and ER chaperone upregulation to assist in protein folding.[4] Typically, the UPR aims primarily at promoting cell survival, but when ER stress is excessive or prolonged, then the goal switches from survival to apoptosis in a caspase-dependent manner.[5]

The UPR is initiated by three ER transmembrane sensors: inositol-requiring enzyme 1α (IRE1α), double stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6).[6,7] The IRE1 pathway is the most ancient branch of the UPR. Under ER stress, IRE1α oligomerizes, autophosphorylates, and excises a 26-nucleotide fragment to generate spliced X-box binding protein1 (XBP1s), which promotes the expression of ER chaperone genes and molecules involved in ERAD.[6,7] IRE1α also acts as a kinase to activate pro-apoptotic c-Jun N-terminal kinase signaling to induce cell death.[6,7] Therefore, IREα plays a paradoxical dual function of pro-survival and pro-apoptotic signaling.

Glycosylation is one of the most common post-translational modifications of proteins in eukaryotic cells. Glycans (*i.e.* carbohydrates or sugars) participate in important biological processes including molecular trafficking, endocytosis and clearance, signal transduction, receptor activation, and cell adhesion.[8] Depending on the linkage type, there are two kinds of protein glycosylation: N- and O-glycosylation.[9] N-Linked glycosylation is the most common form and plays a pivotal role in the folding of glycoproteins in the ER.[9] If newly synthesized polypeptides fail to fold, they are retained in the ER and ultimately degraded by cytoplasmic proteasomes, a process known as ERAD.[10,11] This protein quality control system is important because it prevents accumulation of misfolded proteins in the ER. Accordingly, ERAD is tightly linked with ER stress. The ERAD process involves three functionally distinct steps: recognition of a glycoprotein as misfolded, its

retrotranslocation to the cytoplasm, and subsequent ubiquitin-dependent degradation by the proteasome. The ERAD mechanism is conserved across species, and the hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (HRD1) suppressor enhancer lin12 1-like (SEL1L) complex is essential.[12] HRD1 is a ubiquitin ligase (E3) that is localized in the ER membrane,[13] induced by ER stress,[14] and protects against ER stress–induced apoptosis.[15] On the other hand, SEL1L is a stabilizing factor for HRD1, forms a complex with HRD1, and is induced by ER stress.[14] Consequently, dysfunction of the ERAD system is associated with many disease pathologies.[11]

Granular corneal dystrophy type 2 (GCD2) is an autosomal dominant disorder caused by an arginine to histidine substitution at codon 124 (R124H) in the transforming growth factor β-induced (*TGFBI*) gene.[16] GCD2 is characterized by age-dependent progressive accumulation of deposits of the mutant-*TGFBI* protein (TGFBIp) in the corneal epithelia and stroma, followed by interference with corneal transparency.[17] TGFBIp secretion via the ER/Golgi-dependent secretory pathway is delayed in GCD2 corneal fibroblasts.[18] Defective autophagy, accumulated mutant-TGFBIp,[19-22] delayed TGFBIp secretion,[18,22] and enhanced ER stress[23] are involved in the pathogenesis of GCD2.

Melatonin is not only synthesized by the pineal gland, but is produced by a number of other tissues, such as the skin,[24] eye,[25] bone marrow,[26] gastrointestinal tract,[27] lymphocytes,[28] and thymus;[29] additionally, its receptors have been identified in the eye and may play a role in aqueous humor dynamics.[25,30,31]

Melatonin and its metabolites have received much attention due to their direct free radical scavenging [32-35] and antioxidant properties.[36,37] Numerous studies have documented that melatonin has anti-apoptotic effects on normal cells[38-40] attributable to its antioxidant properties.[41] Melatonin reduces free radical levels via stimulating the activities of antioxidant enzymes.[36,42-46] In addition, melatonin can suppress cell death through reduction of the UPR or ER stress,[47-49] because oxidative stress contributes to activation of the UPR.[35] Nevertheless, the exact molecular mechanisms that underlie the suppressive effects of melatonin on ER stress or the UPR have not been clarified.

Melatonin has been reported to protect against the death of GCD2 corneal fibroblasts from oxidative stress[50] and to enhance the degradation of mutant-TGFBIp via activation of autophagy.[51] Recently, we found that TGFBIp was degraded by the ERAD system under ER stress.[23] In this study, we investigated whether melatonin could modulate activation of the UPR and prevent cell death caused by ER stress in GCD2 corneal fibroblasts, focusing on its regulating mechanism.

2. MATERIALS AND METHODS

2.1 Materials

Tunicamycin, thapsigargin, MG132, bafilomycin A1, dimethyl sulfoxide (DMSO), and melatonin were obtained from Sigma-Aldrich (St Louis, MO, USA). N-Glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA, USA). Anti-TGFBIp (R&D Systems, Minneapolis, MN, USA), anti-β-actin (Sigma-Aldrich), anti-XBP1s (BioLegend, San Diego, CA, USA), Phospho-IRE1α (Ser724; Thermo Scientific, Rockford, IL, USA), and anti-SEL1L (Abcam, Cambridge, UK; *ab78298*) was used to perform western blot. Primary antibodies, caspase-3 (#9662), PARP (#9532), BiP (#3177), IRE1α (#3294), CHOP (#2895), ATF-4 (#11815), ATF-6 (#65880), PERK (#3192), Phospho-PERK (Thr980, #3179), elF2α (#9722), Phospho-elF2α (Ser51, #9721), and the ER Stress Antibody Sampler Kit were purchased from Cell Signaling Technology (Beverly, MA, USA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce (Rockford, IL, USA).

2.2 Corneal fibroblasts and cultivation

Wild-type (WT) (n = 4), GCD2 heterozygous (HT) (n = 1), and GCD2 homozygous (HO) (n = 3) corneal fibroblasts were described previously.[52] Corneal fibroblasts were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 1000 units/mL penicillin, and 1.0 mg/mL streptomycin sulfate (all from Mediatech, Manassas, VA, USA).

2.3 Induction and evaluation of cell death

Melatonin was added 30 min prior to 0.1 μg/mL tunicamycin. Cell death was evaluated 16-72 h after tunicamycin treatment. Antagonism of the melatonin receptors MT1 and MT2 was achieved by adding 10 μM 2-benzyl-N-acetyltryptamine (luzindole) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 30 min before melatonin. Cells were grown in an incubator at 37˚C with 5% CO_2 and 95% humidity.

2.4 Cell treatment

Melatonin was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM and then diluted to a desired concentration directly in the culture medium. Cells treated with DMSO at an equivalent concentration were used as a control.

The dose of melatonin was selected based on previous studies indicating that 0.1–0.3 mM melatonin suppresses cell death from oxidative stress[50] and reduces mutant TGFBIp levels by autophagy activation[51] in GCD2 corneal fibroblasts.

Corneal fibroblasts were assigned to WT and GCD2 groups and were further divided into ER stress groups as follows: control (vehicle, DMSO treatment) and ER stress (tunicamycin treatment) groups. To identify whether melatonin exerts protective effects on ER stress, the treatment groups were as follows: tunicamycin and tunicamycin+melatonin. To investigate the role of melatonin receptor signaling in mediating the protective effects of melatonin, the ER stress group was divided into the following groups: tunicamycin+melatonin and tunicamycin+melatonin+luzindole (a nonselective melatonin receptor antagonist).

2.5 Flow cytometric analysis

Flow cytometry was performed according to manufacturer's protocol (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen, San Diego, CA, USA). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) as described previously.[52]

2.6 RNA isolation and the real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated as described by the manufacturer using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, total RNA was isolated from corneal fibroblasts by extraction in TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Using a Power SYBR® Green RNA-to-CT™ 1-Step kit and StepOnePlus™ (Applied Biosystems, Foster City, CA, USA), we measured mRNA expression by *SEL1L*, *HRD1*, and *ACTIN* according to the manufacturer's instructions. PCR conditions for all genes were as follows: 48°C for 30 min then 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The results were based on cycle threshold (Ct) values. We calculated differences between the Ct values for experimental and reference (*ACTIN*) genes and graphed the results as a percentage of each RNA to the calibrator sample. Primers used were: *SEL1L*: forward, 5′-CTCGCTAACAGGAGGCTCAGTAG-3′; reverse, 5′- GGTGCCACTGGCATGCATCTGAG-3′. *HRD1*: forward, 5′-GCATGGCAGTCCTGTACATCC-3′; reverse, 5′-GCACCATCGTCATCAGGATGG-3′. *ACTIN*: forward, 5′-GGACTTCGAGCAAGAGATGG-3′; reverse, 5′-AGCACTGTGTTGGCGTACAG-3′. mRNA levels were normalized to *ACTIN*.

2.7 XBP1 mRNA splicing assay

Corneal fibroblasts were treated with solvent (dimethyl sulfoxide) or tunicamycin (0.1 μg/mL) with or without melatonin for 3-6 h. Total RNA was isolated as described by the manufacturer using TRIzol reagent. RT-PCR was performed in the same tube (one-step RT- PCR) with the AccuPower RT-PCR PreMix (Bioneer, Seoul, Korea) as described previously.[52] The *XBP1* primers were*:* forward, 5′-TTACGAGAGAAAACTCATGGCC-3′; reverse, 5′-GGGTCCAAGTTGTCCAGAATGC-3′. β-Actin mRNA was used as the internal control. A 283 base pair PCR product was derived from the unspliced

form of *XBP1* mRNA, which contains a 26 base pair intron. A 257 base pair PCR product was derived from the spliced form of *XBP1* mRNA. Thirty PCR amplification cycles were used for *XBP1* and *ACTIN*. The PCR products were separated by electrophoresis on a 2.5% (w/v) agarose gel. The image was revealed by a MiniBIS Pro Gel documentation system (DNR Bio-Imaging Systems, Jerusalem, Israel).

2.8 Preparation of cell lysates and western blot analyses

Cell lysates from cultured corneal fibroblasts were prepared in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris-HCl, pH 7.4) containing protease inhibitors (Complete Mini Protease Inhibitor Tablet, Roche, Indianapolis, IN, USA). Crude cell lysates were centrifuged at 10,000 *g* for 10 min at 4°C to remove nuclear fragments and tissue debris. Total protein concentrations were determined using a bicinchoninic acid assay kit (Pierce). Total cellular proteins were electrophoresed in 10-12.5% Tris-glycine SDS polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% dry milk in TBS-T (0.02 M Tris and 0.15M NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature for 1 h and washed three times with TBS-T. Blots were incubated with primary antibodies. After three washes with TBS-T, blots were incubated with secondary antibodies conjugated to HRP at room temperature for 1 h. HRP-conjugated anti-mouse IgG (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA), anti-rabbit IgG (Amersham Pharmacia Biotechnology), or anti-goat IgG (Pierce) were used as secondary antibodies. Immunoblots were visualized using the enhanced chemiluminescence system (Pierce). Immunoreactive protein bands were scanned and optical densities of the bands were quantified using Image J software, version 1.37 (National Institutes of Health, Bethesda, MD, USA). Scans were corrected by background subtraction, and normalized to the intensity of the corresponding β-actin protein bands.

2.9 Glycosylation analysis

N-Glycosidase F, also known as Peptide-*N*-Glycosidase F (PNGase F), is an amidase of the peptide-N4-(*N*-acetyl-beta-glucosaminyl) asparagine amidase, which specifically removes *N*-linked glycans from glycoproteins. Therefore, we used PNGase F for the glycosylation analysis. Cells were incubated with lysis buffer on ice for 15 min and then clarified by centrifugation at 10,000 *g* for 10 min at 4°C. Samples were deglycosylated using PNGase F following the manufacturer's instructions. Briefly, 2 µL of 10% SDS was added to 15 µL of cell lysate and incubated at 95°C for 5 min. After heating, the samples were incubated on ice for 2 min before the addition of 2 µL G7 buffer, 1 µL 20% octyl β-D-glucopyranoside, and 1 µL PNGase F. Digestion was carried out overnight at 37°C. The samples were then analyzed by western blotting.

2.10 Statistical analysis

Significant differences ($P < 0.05$) between groups and/or treatments were evaluated using Student's t-test or one-way analysis of variance, followed by the Newman-Keuls multiple comparison test. Results are expressed as means ± SDs. All data were processed using Prism version 5.0 graphing analysis software (GraphPad, San Diego, CA, USA).

3. RESULTS

3.1 Melatonin suppress cell death caused by ER stress

We have reported previously that ER stress induces the death of GCD2 corneal fibroblasts.[4] To test the ability of melatonin to protect corneal fibroblasts against ER stress-induced cell death, both WT and GCD2 cultured corneal fibroblasts were treated with 100 μM melatonin for 12-72 h prior to exposure to 0.1 μg/mL tunicamycin. Microscopic images (Fig. 1A-D) indicated that tunicamycin stimulation resulted in significant cell shrinkage and reduced the rate of attachment of GCD2 compared with WT cells. Moreover, microscopy showed that melatonin treatment reduced the cell shrinkage induced by tunicamycin, and increased the rate of cellular attachment (Fig. 1A-D).

Annexin-V/propidium iodide flow cytometric analyses were performed to determine whether the morphological features of cell death were consistent with apoptosis or necrosis. Cells undergoing apoptosis and necrosis are shown in supplementary data 1. Briefly, significant cell death was not observed in either WT or HO cells 12 h after treatment with tunicamycin. However, after 24, 48, and 72 h of treatment, cell death was significantly higher in both cell types than in untreated cells. Treatment of WT and HO cells with both tunicamycin and 100 μM melatonin for 24, 48, and 72 h significantly prevented cell death compared to that observed for cells treated with tunicamycin only. These data are summarized in Table 1.

To confirm the correlation between ER stress and apoptotic cell death, we analyzed the levels of cleaved caspase-3, which is the activated pro-apoptotic form. After a 24 h exposure to tunicamycin, the levels of both cleaved caspase-3 and its cleaved substrate, poly (ADP-ribose) polymerase 1 (PARP1), increased in HO cells (Fig. 1E, right panel). In contrast, melatonin treatment inhibited caspase-3 activity and PARP1 proteolysis induced by tunicamycin in HO cells (Fig. 1E, right panel). We also investigated whether melatonin receptors were involved in the anti-apoptotic mechanism of melatonin against ER stress. Cells were treated with 10 μm luzindole, a melatonin receptor antagonist, 30 min after melatonin treatment. Luzindole abolished the effects of melatonin on caspase-3 and PARP1 (Fig. 1E). Thus, the anti-apoptotic activity of melatonin against ER stress in corneal fibroblasts involved receptor-mediated actions.

Because CHOP is a significant mediator of ER stress-related cell death in GDC2 cells,[23] we investigated whether melatonin could inhibit GCD2 cell death by the suppression of CHOP. Melatonin treatment suppressed the levels of CHOP induced by tunicamycin treatment in both WT and GCD2 cells (supplementary data 2C and J). In addition, tunicamycin induced greater CHOP levels in HO than WT cells (supplementary data 2C), indicating that HO cells are under more ER stress compared to WT cells.

3.2 Melatonin inhibits the UPR activation

To determine the effects of melatonin on regulation of the UPR, WT and HO corneal fibroblasts were co-treated with melatonin and tunicamycin. Tunicamycin enhanced the levels of BiP, IRE1α, and pIRE1α significantly in both WT and HO corneal fibroblasts (Fig. 2A-F). Furthermore, melatonin significantly suppressed the levels of these factors after induction by tunicamycin in WT and HO cells. In addition, because the PERK and ATF6 pathways are also controlled by BiP, we investigated whether melatonin could regulate these pathways and the downstream targets eIF2α and ATF4 under ER stress. Melatonin treatment suppressed the levels of BiP, p-IRE1α, p-PERK, and ATF6 after induction by tunicamycin treatment in both WT and HO corneal fibroblasts (supplementary data 2A, D–G). The phosphorylation of eIF2α and ATF4 were also suppressed by melatonin treatment (supplementary data 2B, H and I).

To investigate the involvement of melatonin receptors in the ability of melatonin to reduce UPR proteins, luzindole was used in combination with melatonin. Luzindole significantly attenuated the effects of melatonin on the levels of BiP, IRE1α, and pIRE1α (Fig. 2D-F). These data suggest that the effects of melatonin on regulating the UPR depend on receptor-mediated signaling.

3.3 Melatonin inhibits splicing of XBP1 induced by ER stress

XBP1 mRNA is spliced by IRE1 in response to ER stress.[53] Therefore, we investigated whether melatonin affected a downstream target of the IRE1α pathway by assessing the splicing of *XBP1* mRNA. The results indicated that the splicing of *XBP1* mRNA was induced by tunicamycin treatment in HO but not WT cells after 3 h (Fig. 3A, lane 6). Furthermore, this splicing was completely inhibited by melatonin (Fig. 3A, D, lane 7). After 6 h of tunicamycin treatment, *XBP1* mRNA was completely spliced in both WT and HO cells (Fig. 3B, lanes 2 and 6). Melatonin treatment completely suppressed the splicing of *XBP1* mRNA in WT cells (Fig. 3B, lane 3). However, in HO cells, splicing of *XBP1* mRNA was only partially suppressed (Fig. 3B, lane 7). These results indicate that HO cells are under ER stress compared to WT cells. Consistent with the effects of melatonin on the splicing of *XBP1* mRNA, tunicamycin treatment induced spliced XBP1 protein in WT (Fig. 3C, lane 2, and 3D, bar 2) and HO cells (Fig. 3C, lane 8, and 3D, bar 8). In addition, melatonin completely inhibited the

levels of spliced XBP protein in both WT (Fig. 3C, lane 3, and 3D, bar 3) and HO cells (Fig. 3C, lane 9, and 3D, bar 9). The inhibitory effects of melatonin on the spliced XBP protein were suppressed significantly by luzindole in both WT and HO cells (Fig. 3 C, D, lanes 4 and 10, and bars 4 and 10).

3.4 The proteasome inhibitor suppress IRE1α reduced by melatonin

Recently, it was reported that one UPR protein, IRE1α, was degraded by ERAD.[54] Therefore, to determine whether reduction of this protein by melatonin was due to ERAD through the proteasome system, cells were treated with the proteasome inhibitor, MG132. The melatonin-induced decrease in IREα was inhibited by MG132 indicating that this change resulted from ERAD activity (Fig. 4A-C, lanes and column 7 compared to 5). Interestingly, MG132 significantly attenuated the inhibitory effects of melatonin on BiP levels induced by tunicamycin (Fig. 4A, C, lane 7 compared with 5). However, the autophagy inhibitor, bafilomycin A1, had no effect on the ability of melatonin to decrease the levels of IRE1α induced by ER stress (Fig. 4A, C, lane 5 compared with 6).

3.5 TGFBIp is degraded via ERAD and melatonin induces ERAD activation

Previously, we demonstrated that, under ER stress conditions, TGFBIp could be degraded by the ERAD system.[23] Therefore, to determine whether melatonin regulated TGFBIp levels through this system, cells were co-treated with or without melatonin and MG132 under ER stress. Tunicamycin co-treatment with MG132 or bafilomycin A1 significantly upregulated TGFBIp levels under ER stress compared to tunicamycin treatment only (Fig. 4D, E, lanes 2 and 3 compared to lane 1). Melatonin treatment reduced the TGFBIp level compared to tunicamycin treatment only (Fig. 4D, E, lane 4 compared to 1). MG132 and bafilomycin A1 suppressed the inhibitory effects of melatonin on TGFBIp levels under ER stress (Fig. 4D, E, lane 6 compared to 4, and lane 5 compared to lane 4, respectively). However, tunicamycin had no significant effects on TGFBIp levels in cells treated with bafilomycin A1 (Fig. 4D, E, lane 2 compared to 7). Furthermore, TGFBIp was increased significantly in cells co-treated with tunicamycin, melatonin, and bafilomycin A1 (Fig. 4D, E, lane 5 compared to 2).

3.6 Decreased levels of SEL1L in GCD2 corneal fibroblasts

TGFBIp is degraded by the ERAD system[23] and mutant-TGFBIp accumulates in GCD2 corneal cells. HRD1 and SEL1 are components of ERAD, which is a retrograde transport mechanism from the ER to the cytosol for eliminating misfolded proteins. Thus, we investigated whether the ERAD system was involved in the pathogenesis of GCD2. RT-PCR analyses showed that HRD1 mRNA was reduced significantly in GCD2 ($0.762 \pm 0.008.154$) compared to WT cells (1.107 ± 0.053) ($P < 0.05$) (Fig. 5A). SEL1L mRNA was also reduced significantly in GCD2 (0.655 ± 0.098) compared to WT cells (0.105 ± 0.085) ($P < 0.05$) (Fig. 5A). Furthermore, the levels of SEL1L protein were reduced significantly in GCD2 (4.862 ± 0.943) compared to WT cells (9.355 ± 0.555) ($P < 0.003$) (Fig. 5B, C).

3.7 Melatonin increases SEL1L levels

Activation of the IRE1α-XBP1 signaling pathway induces the ERAD system by enhancing the expression of HRD1 and SEL1.[55] Therefore, the melatonin-reduced IRE1α and spliced XBP1 levels (Fig. 3A, C) indicate the possibility that melatonin might be involved with regulating the ERAD system. To explore this possibility, we investigated the levels of SEL1L in cells treated without melatonin or with melatonin at various doses. Surprisingly, melatonin increased SEL1L protein levels in a dose-dependent manner (0.1–0.3 mM) after 16 h in both WT and HO cells (Fig. 6A). However, the upregulation of *SEL1L* at 0.3 mM tended to decrease at 0.5 mM melatonin in both WT and HO cells. Melatonin (0.3 mM) significantly increased the level of SEL1L in both WT and HO cells (Fig. 6B).

Despite melatonin inhibiting activation of the IRE1α-XBP1 signaling pathway, the ER stress-inducible protein SEL1 was induced by melatonin. To investigate the basis of this discrepancy, we examined the effects of melatonin on the regulation of SEL1L under ER stress. Tunicamycin (Fig. 6C, lane 3) but not thapsigargin (Fig. 6C, lane 2) treatment shifted the electrophoretic migration of SEL1L. This indicated that tunicamycin prevented deglycosylation of SEL1L. Melatonin treatments suppressed the shift in migration of the SEL1L protein (Fig. 6C, lane 4 compared to lane 3) and deglycosylation of SEL1L protein by tunicamycin in a dose-dependent manner (Fig. 6D). These results indicate that the increased SEL1L levels following treatment with melatonin may result from increased N-linked glycosylation rather than increased gene expression.

Because SEL1 is a well-known N-linked glycoprotein,[56] the mechanism underlying the effects of melatonin were further clarified. Melatonin-treated cell lysates were incubated with or without PNGase F, an enzyme that catalyzes the complete removal of N-linked oligosaccharide chains from glycoproteins. Interestingly, PNGase F treatments showed that levels of SEL1L were unchanged between the melatonin- and no-treatment cell lysates (Fig. 6E, F). Taken together, these results suggest that melatonin enhanced N-linked glycosylation on the SEL1L protein.

4. DISCUSSION

There is increasing evidence that cell death triggered by ER stress is involved in the pathogenesis of a number of diseases including GCD2, and that this process is a target for therapeutic intervention. As a potential therapeutic agent for GCD2, we considered that melatonin would reduce the burden of unfolded or misfolded mutant-TGFBIp in the ER and/or block the UPR signaling pathways that trigger harmful downstream events.

Numerous studies have demonstrated that prolonged ER stress is involved in the process of apoptosis and that melatonin prevents this cell death[57,58]. However, the molecular mechanisms by which melatonin reduces ER stress-induced apoptosis have not been clarified in GCD2. In this study, we showed that melatonin inhibited cell death induced by tunicamycin in GCD2 corneal fibroblasts. Specifically, melatonin significantly inhibited the activation of caspase-3 and cleavage of PARP1 induced by tunicamycin. In addition, tunicamycin treatment elicited more apoptosis of GCD2 corneal fibroblasts compared to WT cells. These results indicate that melatonin prevents apoptosis caused by ER stress and that GCD2 corneal fibroblasts are more sensitive to ER stress compared to WT cells.

Melatonin significantly attenuated the tunicamycin-induced upregulation of BiP, a central regulator of ER stress, due to its ability to control activation of the UPR system.[59] BiP is involved in many cellular processes, including translocating newly synthesized polypeptides, facilitating the folding and assembly of proteins, and targeting misfolded proteins for ERAD.[59-61] Upon ER stress, BiP activates these UPR signaling pathways through the release of ER signal transducers such as PERK, IRE1α, and ATF6.[59] Consequently, attenuation of ER stress by melatonin may be initiated by a reduction in the BiP level. However, questions remain whether melatonin can directly reduce BiP levels.

Melatonin inhibited both the activation of IRE1α and the splicing of XBP1, effects that are implicated in cell death when ER stress is inadequately resolved.[62] Sustained XBP1 splicing induces apoptosis[63] and melatonin inhibits cell death by the suppression of CHOP, which is a significant mediator of ER stress-related cell death in GCD2 (supplementary data 2C). Accordingly, we propose that the IRE1α-XBP pathway, a UPR signaling pathway, may play a role in the anti-apoptotic effects of melatonin in GCD2 cells under ER stress, although melatonin also reduced other PERK and ATF6 signaling pathways controlled by BiP (supplementary data 2A, F and G).

Interestingly, melatonin reduced TGFBIp levels under ER stress. Furthermore, treatment with MG132, an ERAD inhibitor, significantly suppressed the effects of melatonin on TGFBIp levels under ER stress. Therefore, because TGFBIp is degraded by the ERAD system under ER stress,[23] melatonin may activate the ERAD system, which could reduce BiP levels by removing accumulated mutant TGFBIp in the ER lumen. A number of studies have focused on the accumulation of mutant TGFBIp in both the extracellular matrix and cells,[18,19] but the molecular mechanisms leading to this accumulation remain unknown. We hypothesized that defective ERAD in GCD2 corneal fibroblasts was one possible mechanism of mutant TGFBIp accumulation. Knockdown of SEL1L reduces HRD1

levels,[64] while high SEL1L levels activate ERAD through stability with HRD1. These data indicate that the ERAD system in GCD2 cells is defective, and this abnormality could induce accumulation of mutant TGFBIp. Furthermore, a deficiency of SEL1L disrupts ER homeostasis, induces ER stress, impairs ERAD, inhibits protein secretion, attenuates translation, and promotes cell death.[65,66] These findings could explain the pathophysiology of GCD2 which is associated with increased ER stress,[23] accumulated mutant TGFBIp,[19,67] and delayed mutant TGFBIp secretion[18] in corneal fibroblasts. Consequently, we speculate that reduced SEL1L levels may play a pivotal role in ER stress, and exacerbate the pathophysiology in GCD2 corneal fibroblasts.

We confirmed that GCD2 corneal cells contained reduced levels of SEL1L. Even more importantly, we showed, for the first time, that melatonin treatment promoted the biosynthesis or processing of N-linked glycosylation of the SEL1L protein in corneal fibroblasts, thereby elevating SEL1L levels. Melatonin also suppressed the inhibition of glycosylation induced by tunicamycin, an inhibitor of N-acetylglucosamine transferases, which blocks the addition of asparagine N-linked glycans to glycoproteins.[68] Accordingly, these data support the possibility that melatonin might modulate the activity of the N-acetylglucosamine transferases.

N-Glycosylation of proteins in the ER is critical for protein quality control.[69] The results of the current study indicate the possibility that melatonin could reduce ER stress through the promotion of glycosylation of newly synthesized proteins in the ER lumen. N-Glycans contribute to proper protein folding, assembly, and stability due to their physiological properties, and to quality control through targeting the ERAD.[70] Based on these findings, we speculate that melatonin-enhanced glycosylation on newly synthesized proteins in the ER offers the opportunity for these proteins to pass through the ER quality control machinery. Thus, melatonin may alleviate the protein load in the ER thereby reducing ER stress and/or UPR signaling.

There are unanswered questions about whether melatonin promotes glycosylation of all glycoproteins, which are approximately half of all human proteins, most of which contain N-glycan structures.[71] It is possible that melatonin could enhance the N-glycosylation of other proteins. This notion is supported by our preliminary data that melatonin increased the levels of several glycoproteins (unpublished data). In future studies, we will investigate further whether melatonin can induce N-linked glycosylation of other proteins.

Altered N-glycosylation occurs in many pathological states, and genetic defects in glycosylation may cause various diseases.[72] Therefore, understanding how N-glycosylation is regulated is an important issue.[73] Because N-glycosylation affects protein folding, oligomerization, quality control, sorting, and transport,[70,74] the results of the current study suggest that the suppressive mechanism of melatonin on ER stress could result from reducing the ER protein load by promoting N-glycosylation of newly synthesized glycoproteins. This mechanism provides new perspectives on the physiological function of melatonin.

In GCD2 cells, reduced SEL1 levels were due to changes in the mRNA levels, indicating transcriptional regulation. This notion was supported by an experiment showing that administration of PNGase F, an inhibitor of N-glycosylation, had no effect (unpublished data). However, additional research on the regulation of *SEL1L* in GCD2 cells is an important area for future study.

The effects of melatonin may be mediated through G protein-coupled receptors such as MT1, MT2, and MT3.[75] Luzindole was the first competitive melatonin receptor antagonist discovered.[76] In the current study, luzindole significantly suppressed the effects of melatonin on the cleavage of caspase-3 and PARP1, and on reducing the ER chaperones BiP, IRE1α, and XBP1s. These results suggest that these effects resulted from the melatonin receptor-mediated signal transduction pathway. In addition, although melatonin is produced mainly by the pineal gland, it is also synthesized by the retina,[77] lens,[78] and ciliary body[79] of the eye. Based on our previous data that corneal fibroblasts express MT1 and MT2, which were significantly higher in GCD2 than in WT cells,[50] we speculate that melatonin metabolism may play a role in corneal physiology. Further studies are needed to clarify the details of the physiological roles of melatonin in the eye, as well as the potential therapeutic effects in other ocular diseases.

The effects of melatonin have been examined in previous studies to identify potential therapeutic agents for GCD2. First, GCD2 cells are highly susceptible to oxidative stress-induced cell death[50] and melatonin treatment protects WT and GCD2 cells from oxidative stress by direct free radical scavenging and the upregulation of several antioxidant enzymes via its receptors.[50] Second, mutant TGFBIp accumulates in autophago-lysosomes by impaired autophagic flux in GCD2 corneal fibroblasts. Growing evidence suggests that oxidative stress induces the intracellular accumulation of proteins that cause age-related diseases, including Alzheimer's disease.[80] These results have led to the hypothesis that melatonin could specifically reduce the accumulation of mutant-TGFBIp via the reduction of oxidative stress and/or the activation of autophagic degradation. In fact, we have demonstrated that melatonin reduces mutant-TGFBIp via the activation of autophagy.[51] Finally, we have recently reported that ER stress and ERAD abnormalities are involved in the pathogenesis of GCD2. Interestingly, TGFBIp can be degraded by the activation of ERAD. Furthermore, treatment with 4-PBA, which reduces ER stress, significantly reduced the levels of TGFBIp via the activation of ERAD.[23] In addition, melatonin can reduce ER stress.[23] Based on these results, we hypothesized that, similar to 4-PBA, melatonin reduces ER stress and TGFBIp via the activation of ERAD. Taken together, our results suggest that melatonin has potential therapeutic effects for GCD2. Further investigations are needed for drug development for TGFBI-linked corneal dystrophy using animal models.

Lithium,[81] 4-PBA[23] and melatonin[50,51] are suggested therapeutic agents in TGFBI-linked corneal dystrophy including GCD2. Common therapeutic effects of these agents include the reduction of TGFBIp levels in GCD2. However, because the cornea is characterized by lipophilic and hydrophilic structures, it is a barrier to the absorption of both hydrophilic and lipophilic

molecules.[82,83] Therefore, ophthalmological drug delivery is one of the most challenging endeavors facing pharmacologists. In the case of lithium, despite its ability to reduce the level of TGFBIp by the activation of autophagy and inhibition of the TGF-β signaling pathway according to in vitro analyses,[81] it could not penetrate the cornea in rabbit eyes (unpublished data). This property could be a disadvantage with respect to GCD2 therapy. Previous studies have demonstrated that melatonin and 4-PBA could also reduce the levels of mutant-TGFBIp by the activation of autophagy[51] and ERAD,[23,50] respectively. Taken together, melatonin could reduce TGFBIp via two degradation pathways, autophagy and ERAD. This could be an obvious advantage of melatonin compared to 4-PBA as a therapeutic agent in TGFBI-linked corneal dystrophy. Another advantage of melatonin and 4-PBA is the potential for delivery to eye tissues. Melatonin readily passes biological membranes and exerts anti-oxidative activity at both the cell membrane and in the cells.[84] 4-PBA also efficiently penetrates the cornea by treatment with topical eye drops.[84] Additionally, like melatonin, 4-PBA could suppress apoptotic GCD2 cell death from ER stress.[23,50] However, a major difference is that while 4-PBA is a small molecule chemical, melatonin is a hormone synthesized in the eye, including the corneal ciliary body.[79] These results indicate that melatonin has advantages compared to 4-PBA with respect to therapeutic applications because melatonin synthesis in these tissues can be regulated by a circadian clock, *etc*. Finally, while 4-PBA is approved by the US FDA, melatonin is not. However, melatonin is available as a food supplement and approval from the US FDA for use as a drug has not been required. Therefore, there may not be safety issues with respect to the use of melatonin as a therapeutic agent. Taken together, both melatonin and 4-PBA are attractive drug candidates in TGFBI-linked corneal dystrophy including GCD2. Additionally, we speculate that despite the observation that melatonin reduced TGFBIp in vitro, this potential weakness does not preclude its clinical therapeutic applications. Therefore, additional studies are needed to elucidate the enhanced effects of the combination of melatonin and 4-PBA as a therapeutic approach for GCD2.

In conclusion, the results of this study reveal novel mechanisms by which melatonin can reduce ER stress, and may activate the ERAD system through promotion of N-glycosylation on the SEL1L protein. Furthermore, melatonin promotes degradation of mutant TGFBIp through activation of ERAD. Additionally, melatonin suppresses the caspase-3 activation pathway, thereby protecting cells from apoptosis induced by ER stress. There are no current pharmacological therapeutic drugs for TGFBI-linked corneal dystrophy, and melatonin is a potential new approach for the treatment of GCD2 and other diseases related to ER stress.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supplementary data 1. Flow cytometric analyses of corneal fibroblasts apoptosis after treatment with vehicle (dimethyl sulfoxide, DMSO) or tunicamycin (Tm, 0.1 mM) with or without 100 μM melatonin for (A) 12, (B) 24, (C) 48, or (D) 72 h.

Supplementary data 2. Melatonin modulates the UPR in corneal fibroblasts.

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Figure legends

FIGURE 1 Melatonin prevents apoptotic death of GCD2 cells caused by an ER stress inducer. Phase-contrast photomicrographs of corneal fibroblasts treated with vehicle (dimethyl sulfoxide, DMSO) or tunicamycin (Tm, 0.1 mM) with or without 100 μM melatonin for (A) 12, (B) 24, (C) 48, or (D) 72 h (magnification: 320×). (E) Caspase-3, poly (ADP-ribose) polymerase 1 (PARP1), and β-actin were examined by western blotting in wild-type (WT) and homozygous (HO) corneal fibroblasts treated with or without the indicated concentrations of Tm, melatonin, and/or luzindol (Luzin).

FIGURE 2 Melatonin significantly inhibits the levels of BiP and IRE1α induced by ER stress in wild-type (WT) and homozygous (HO) corneal fibroblasts. (A-C) Cells were treated with dimethyl sulfoxide (DMSO), tunicamycin (Tm) (1 μg/mL), and/or melatonin (Mel) (1 mM) for 16 h. Cells were lysed and underwent western blotting. Protein levels were normalized to the β-actin protein signal. Densitometric analyses indicate that Tm treatment upregulates BiP (B, lanes 2 and 6) and IRE1α (C, lanes 2 and 6) expression significantly. Mel alone has no effect on BiP (A and B, lanes 4 and 8) and IRE1α (A and C, lanes 4 and 8). Mel suppresses the levels of BiP and IRE1α induced by Tm (A, B, and C, lanes 3 and 7). (D-F) Western blot analyses of IRE1α, p-IRE1α, and β-actin (50 μg of total proteins) from WT and HO corneal fibroblasts. WT corneal fibroblasts were pre-incubated for 30 min with or without 10 μm luzindole (Luzin) and exposed to 0.3 mM Mel for 16 h. Levels of IRE1α and p-IRE1α are increased in response to Tm treatment (D, E, and F, lanes 2 and 8). Combined treatment of Tm with melatonin results in significantly reduced levels of IRE1α (E, lanes 3 and 8) and p-IRE1α

(F, lanes 3 and 8) compared to DMSO. Melatonin and Luzin alone have no effect on IRE1α and p-IRE1α levels (D, E, and F). However, Luzin treatment significantly suppresses the levels of IRE1α and p-IRE1α induced by tunicamycin (D, E, and F, lanes 4 and 10). Results are reported as the mean percentages of non-treatment ± SD. *P < 0.05 compared to the Tm alone treatment group.

FIGURE 3 Melatonin significantly inhibits alternative splicing of XBP1 induced by tunicamycin (Tm). Corneal fibroblasts were treated with or without Tm (5 μg/mL) and/or luzindole (Luzin) (10 μM) in combination with melatonin (Mel) (0.3 mM) for (A) 3 and (B) 6 h. Total RNA was extracted and *XBP1* and *β-ACTIN* mRNAs were evaluated by RT-PCR. (A) Splicing of *XBP1* mRNA is induced by Tm in homozygous (HO) (lane 6), but not wild-type (WT) cells after 3 h (lane 2). This splicing is completely inhibited by Mel (lane 7). (B) After 6 h, *XBP1* mRNA is completely spliced in both WT and HO cells (lanes 2 and 6). Mel completely suppresses the splicing of *XBP1* mRNA in WT cells (lane 3). However, splicing of *XBP1* mRNA in HO cells is only partially suppressed (lane 7). Whole-cell lysates were subjected to western blotting using anti-XBP1 and anti-actin antibodies. Mel completely inhibits the levels of spliced XBP protein in both WT (C, lane 3, and D, bar 3) and HO cells (C, lane 9, and D, bar 9). The inhibitory effects of Mel on the spliced XBP protein were significantly suppressed by Luzin in both WT and HO cells (C and D, lanes 4 and 10, and bars 4 and 10). XBP1 protein levels were normalized to the β-actin protein signals. *P < 0.05 with versus without melatonin.

FIGURE 4 BiP, IRE1α, and TGFBIp are degraded by ER-associated protein degradation (ERAD), and melatonin (Mel) stimulates ERAD activity. (A) TGFBIp and BiP levels in wild-type (WT) and homozygous (HO) corneal fibroblasts cultured for 18 h in the presence of tunicamycin (Tm) (0.1 mM) with and without MG132 (1 mM), bafilomycin A1 (Baf A1) (0.1 nM), and Mel (0.3 mM). Whole-cell lysates were subjected to western blotting using anti-BiP, anti-IRE1α, and anti-actin antibodies. The densitometric results were normalized to β-actin. (B) Densitometric analysis of BiP levels. (C) Densitometric analysis of IRE1α levels. MG132 significantly attenuates the inhibitory effects of Mel on IREα and BiP levels induced by Tm (A, B, and C, lane 7 compared with lane 5). Baf A1 has no effects on the inhibitory effects of Mel on the levels of IRE1α and BiP induced by ER stress (A, B, and C, lane 5 compared with lane 6). (D) TGFBIp levels assayed by western blot in WT and HO cells using an anti-TGFBIp antibody. (E) Densitometric analysis of TGFBIp levels. Tm co-treatments with MG132 or Baf A1 upregulated the levels of TGFBIp under ER stress compared to Tm only (D and E, lanes 2 and 3 compared to lane 1). Mel reduces the level of TGFBIp compared to Tm only (A and B, lane 4 compared to lane 1). MG132 and Baf A1 suppress the inhibitory effects of melatonin on TGFBIp levels under ER stress (D and E, lane 6 compared to lane 4, and lane 5 compared to lane 4). *P < 0.05 compared to the Tm only treatment group.

FIGURE 5 Alterations of the ER-associated protein degradation (ERAD) system in GCD2 corneal fibroblasts. (A) RT-PCR analyses of *HRD1* and *SEL1L* mRNA in wild-type (WT) and homozygous (HO) corneal fibroblasts. *HRD1* and *SEL1L* mRNA levels are reduced significantly in HO compared to WT cells. *HRD1* and *SEL1L* mRNA levels were normalized to the level of β-actin mRNA (*p < 0.05). (B) SEL1L protein levels. Total cell lysates were analyzed by western blot using an anti-SEL1L antibody. The level of SEL1L protein is reduced significantly in HO compared to WT cells. (C) Densitometric analysis of SEL1L normalized to β-actin levels. Data are presented as means ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$.

FIGURE 6 Melatonin (Mel) regulates the ER-associated protein degradation (ERAD) system. (A) Western blot analyses of SEL1L and β-actin in 50 μg of total proteins from wild-type (WT) and homozygous (HO) corneal fibroblasts treated with or without 100, 200, or 300 μM Mel for 16 h. (B) SEL1L levels after normalization to β-actin protein signals in WT (blue) and HO (red) corneal fibroblasts. (C) SEL1L was analyzed by western blot in WT corneal fibroblasts treated with thapsigargin (Tg), tunicamycin (Tm), and/or Mel. Mel treatment suppresses the reduction of N-glycosylation of SEL1L by Tm. (D) WT corneal fibroblasts co-treated with or without 100, 200, or 300 μM Mel and Tm (0.1 μg/mL) for 16 h. SEL1L was analyzed by western blot using an anti-SEL1 antibody. Mel inhibits deglycosylation caused by Tm in a dose-dependent manner. (E) Mel promotes N-glycosylation on SEL1L. WT corneal fibroblasts treated with or without 100, 200, or 300 μM Mel for 16 h. Mel treated-cell lysates were treated with PNGase F and the levels of SEL1L analyzed by western blot. Lysates with (+) or without (-) PNGase F treatment are indicated below each blot lane. (F) Densitometric analysis of SEL1L levels normalized to β-actin levels. All data are expressed as mean percentages of control ± SD of three independent experiments. *$P < 0.05$ compared to the control group. Short arrows indicate unglycosylated SEL1L protein. Long arrows indicate glycosylated SEL1L forms.

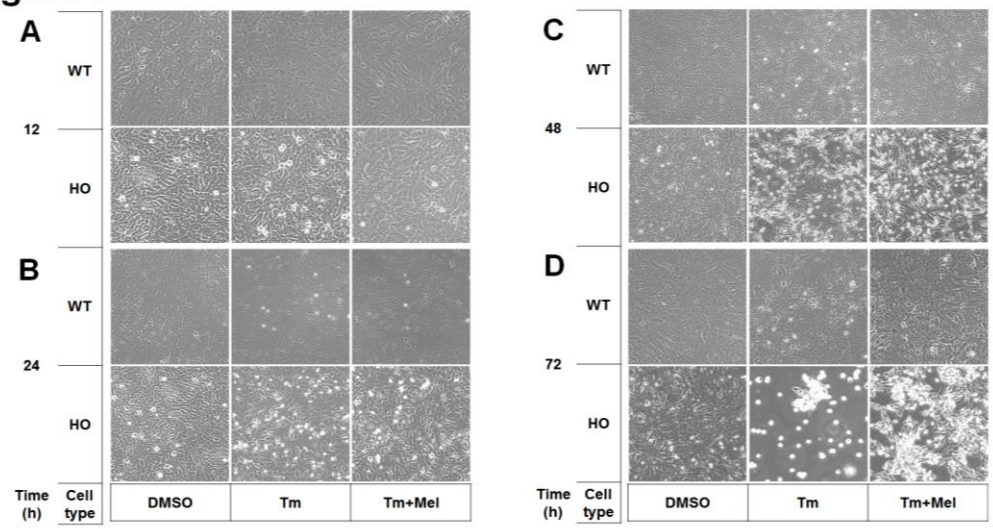
FIGURE 7 Schematic of the mechanism proposed for the effects of melatonin on ER stress and GCD2 pathogenesis. Defective ERAD and reduced SEL1L levels may result in the accumulation of proteins including mutant-TGFBIp in the ER lumen. This can induce ER stress and the unfolded protein response (UPR), followed by IRE1α activation and XBP1 splicing. Subsequently, defective ERAD would affect the pathogenesis of ER stress-related diseases including GCD2. Melatonin stimulates N-glycosylation of newly synthesized glycoproteins and ERAD activity through enhancing the stability between SEL1L and HRD1. This reduces ER stress and subsequent UPR signaling of IRE1α activation and XBP1 splicing. Melatonin protects GCD2 cells from death through the reduction of ER stress.

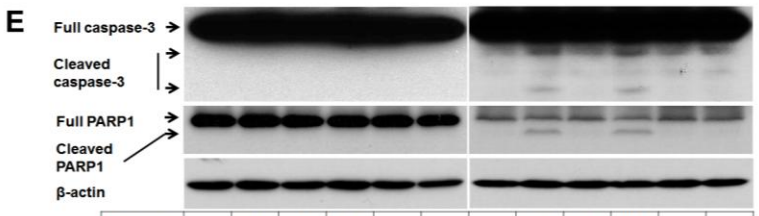
Table 1 Effect of melatonin on tunicamycin-induced cell death

Cell Death (%)						
Cell type		WT			HO	
Time after treatment	Vehicle	0.1 μg/mL Tm Vehicle	0.1 μg/mL Tm 100 μM Mel	Vehicle	0.1 μg/mL Tm Vehicle	0.1 μg/mL Tm 100 μM Mel
6 h	3.40 ± 0.26	4.50 ± 0.40	3.73 ± 0.32	3.72 ± 0.66	5.13 ± 0.30	3.96 ± 0.75
24 h	3.10 ± 0.19	6.19 ± 2.76	3.80 ± 0.36*	4.73 ± 1.62	8.54 ± 0.71	6.22 ± 1.06*
48 h	3.73 ± 0.40	6.53 ± 0.84	4.50 ± 0.19*	4.25 ± 0.24	41.00 ± 1.00	26.00 ± 2.00*
72 h	3.50 ± 0.40	10.63 ± 0.63	6.16 ± 0.56*	8.80 ± 1.04	67.33 ± 2.08	54.87 ± 3.64*

The percentages of WT and HO cells undergoing apoptosis and necrosis were measured using fluorescence-activated cell sorting. WT, wild-type; HO, GCD2 homozygous; Mel, melatonin; Tm, tunicamycin; The vehicle was dimethyl sulfoxide. Results represent the means ± SD of 3 independent experiments. *$P < 0.05$, HO versus WT cells.

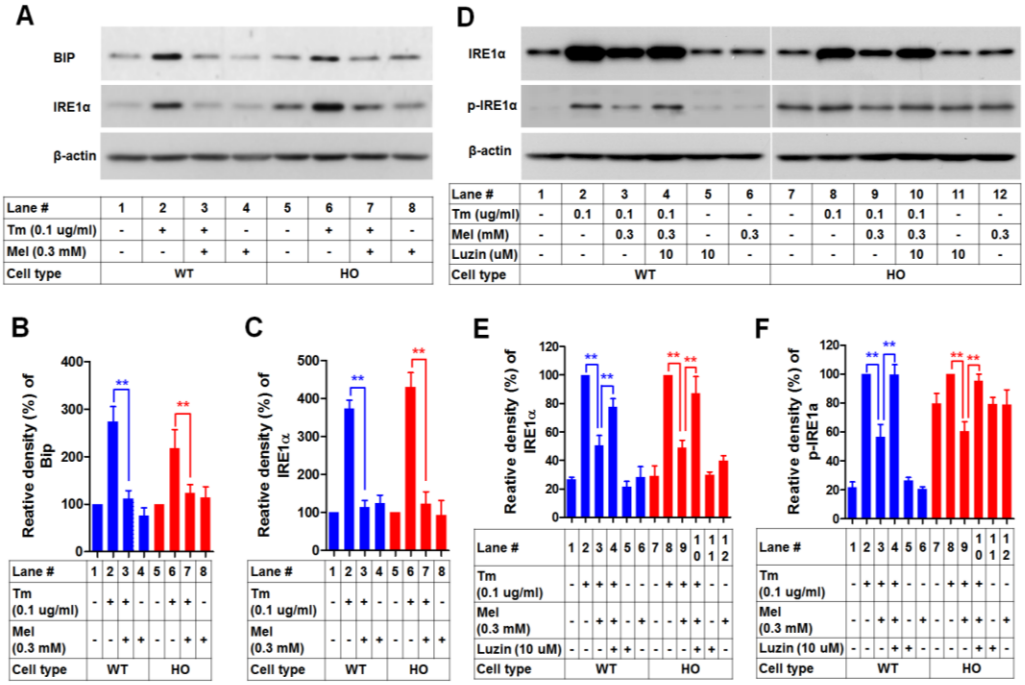
Figure 1





Tm (μg/ml)	-	0.1	0.1	0.1	-	-	-	0.1	0.1	0.1	-	-
Mel (mM)	-	-	0.3	0.3	-	0.3	-	-	0.3	0.3	-	0.3
Luzin (μM)	-	-	-	10	10	-	-	-	-	10	10	-
Cell type	WT						HO					

Figure 2



Lane #	1	2	3	4	5	6	7	8
Tm (0.1 ug/ml)	-	+	+	-	-	+	+	-
Mel (0.3 mM)	-	-	+	+	-	-	+	+
Cell type	WT				HO			

Lane #	1	2	3	4	5	6	7	8	9	10	11	12
Tm (ug/ml)	-	0.1	0.1	0.1	-	-	-	0.1	0.1	0.1	-	-
Mel (mM)	-	-	0.3	0.3	-	0.3	-	-	0.3	0.3	-	0.3
Luzin (uM)	-	-	-	10	10	-	-	-	-	10	10	-
Cell type	WT						HO					

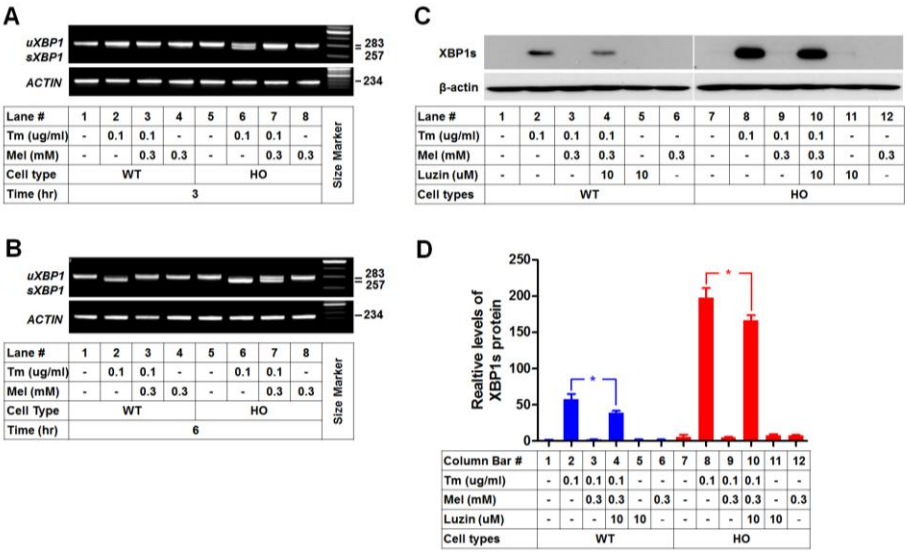
Lane #	1	2	3	4	5	6	7	8
Tm (0.1 ug/ml)	-	+	+	-	-	+	+	-
Mel (0.3 mM)	-	-	+	+	-	-	+	+
Cell type	WT				HO			

Lane #	1	2	3	4	5	6	7	8
Tm (0.1 ug/ml)	-	+	+	-	-	+	+	-
Mel (0.3 mM)	-	-	+	+	-	-	+	+
Cell type	WT				HO			

Lane #	1	2	3	4	5	6	7	8	9	10	11	12
Tm (0.1 ug/ml)	-	+	+	+	-	-	-	+	+	+	-	-
Mel (0.3 mM)	-	-	+	+	-	+	-	-	+	+	-	+
Luzin (10 uM)	-	-	-	+	+	-	-	-	+	+	-	-
Cell type	WT						HO					

Lane #	1	2	3	4	5	6	7	8	9	10	11	12
Tm (0.1 ug/ml)	-	+	+	+	-	-	-	+	+	+	-	-
Mel (0.3 mM)	-	-	+	+	-	+	-	-	+	+	-	+
Luzin (10 uM)	-	-	-	+	+	-	-	-	+	+	-	-
Cell type	WT						HO					

Figure 3



Lane #	1	2	3	4	5	6	7	8	Size Marker
Tm (ug/ml)	-	0.1	0.1	-	-	0.1	0.1	-	
Mel (mM)	-	-	0.3	0.3	-	-	0.3	0.3	
Cell type	WT				HO				
Time (hr)	3								

Lane #	1	2	3	4	5	6	7	8	9	10	11	12
Tm (ug/ml)	-	0.1	0.1	0.1	-	-	-	0.1	0.1	0.1	-	-
Mel (mM)	-	-	0.3	0.3	-	0.3	-	-	0.3	0.3	-	0.3
Luzin (uM)	-	-	-	10	10	-	-	-	-	10	10	-
Cell types	WT						HO					

Lane #	1	2	3	4	5	6	7	8	Size Marker
Tm (ug/ml)	-	0.1	0.1	-	-	0.1	0.1	-	
Mel (mM)	-	-	0.3	0.3	-	-	0.3	0.3	
Cell Type	WT				HO				
Time (hr)	6								

Column Bar #	1	2	3	4	5	6	7	8	9	10	11	12
Tm (ug/ml)	-	0.1	0.1	0.1	-	-	-	0.1	0.1	0.1	-	-
Mel (mM)	-	-	0.3	0.3	-	0.3	-	-	0.3	0.3	-	0.3
Luzin (uM)	-	-	-	10	10	-	-	-	-	10	10	-
Cell types	WT						HO					

Figure 4

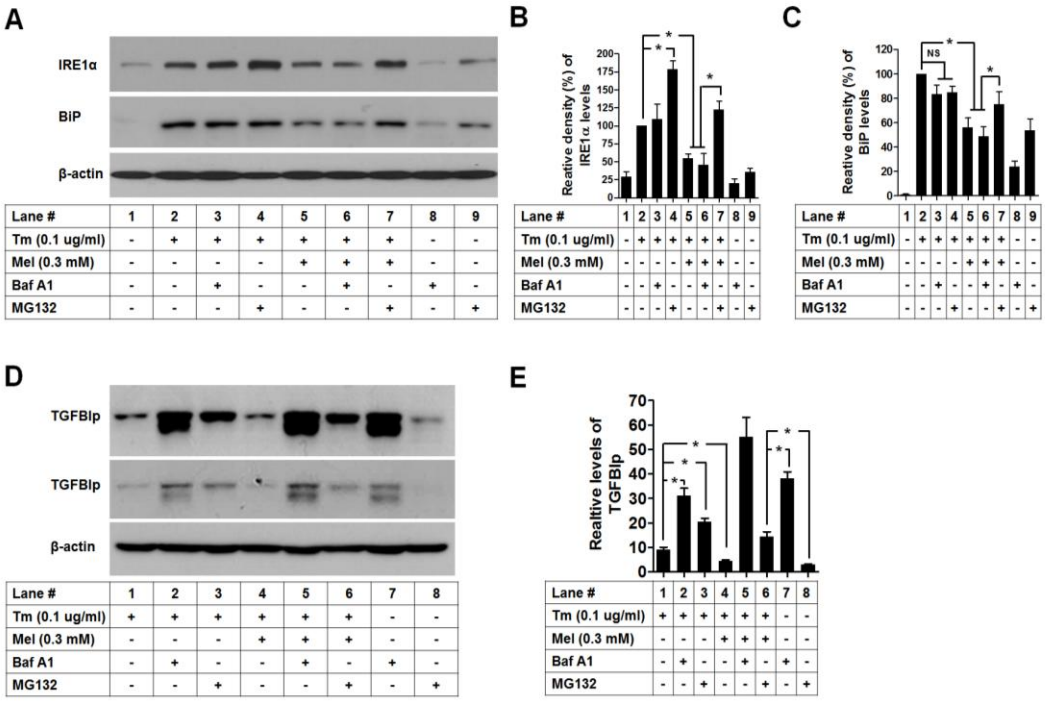


Figure 5

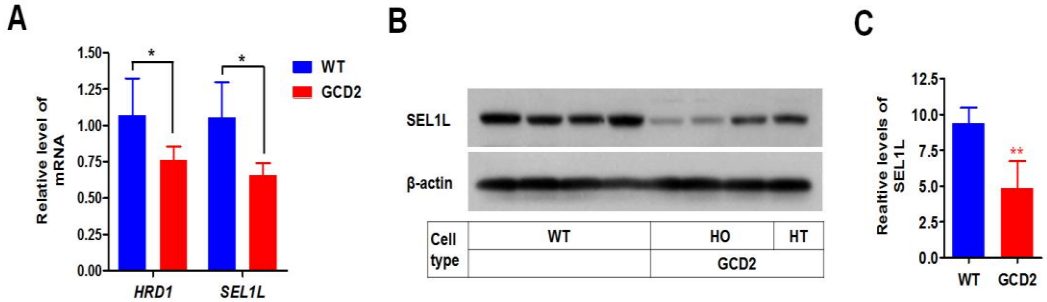


Figure 6

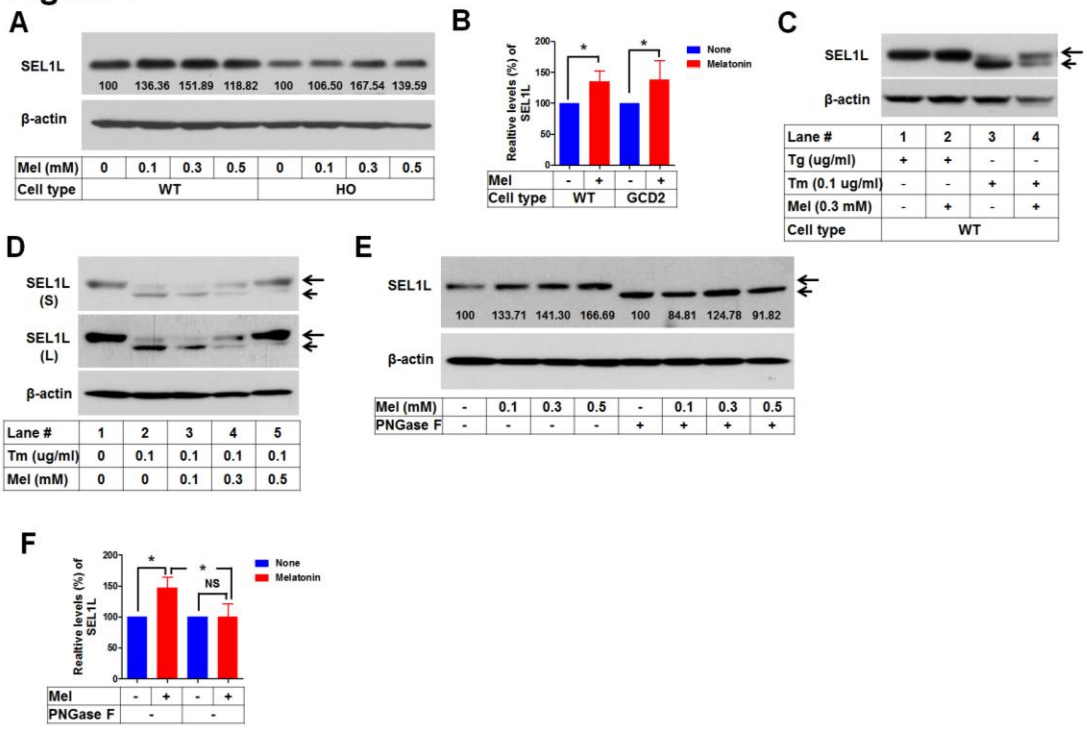


Figure 7