Nucleobindin 1 Controls the Unfolded Protein Response by Inhibiting ATF6 Activation*§

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The endoplasmic reticulum (ER)3 is the critical compartment for modification, folding and assembly of newly synthesized secretory and transmembrane proteins. The ER functions are often affected by such stress conditions as nutrient starvation, decrease in folding efficiency, and overload of protein synthesis that lead to the accumulation of misfolded or unfolded proteins in the ER (1). To cope with ER stress, cells activate a signal transduction pathway, which is called the unfolded protein response (UPR). The UPR consists of three major components: 1) transcriptional induction of ER-resident molecular chaperones and folding enzymes, such as glucose-regulated protein 78, 94 (GRP78, GRP94) and protein-disulfide isomerase (PDI), 2) translational attenuation to prevent further loading of proteins into the ER, and 3) ER-associated degradation (ERAD) to clear misfolded proteins. These adaptive responses are sensed and initiated by ER transmembrane proteins, ATF6, PKR-like ER kinase (PERK) and inositol-requiring 1 (IRE1) (1).

ATF6 is a bZIP transcription factor that plays a major role in UPR transcriptional induction via binding to the ER stress response elements (ERSE: CCAAT-N9-CCACG, ERSE-II: ATTGG-N-CCACG, UPRE: TGACGTGG/A) in the promoter of target genes (2–6). During ER stress, ATF6 is activated by regulated intramembrane proteolysis (RIP), which is also known for its activation mechanism of sterol regulatory element-binding proteins (SREBPs) (7). RIP is defined as the process by which transmembrane proteins are cleaved to release cytosolic domains that function as the transcription factor (7). ATF6 is, in fact, a type-II ER transmembrane protein with the N-terminal domain facing the cytosol and its C terminus in the ER lumen, and the cytosolic N-terminal domain contains DNA-binding and a transcription-activating domain (8). Under normal conditions, ATF6 is kept inactive by binding with GRP78 in the ER lumen. When unfolded or misfolded proteins accumulate in the ER, GRP78 dissociates from ATF6 to repair these unfolded or misfolded proteins (9). Consequently, ATF6 is able to translocate from the ER to the Golgi apparatus. Then, it is converted to the active form (p50ATF6) via cleavage by site-1 protease (S1P) and subsequently by site-2 protease (S2P). The active p50ATF6 is released into the cytosol and translocates to the nucleus, resulting in transcriptional activation (10, 11).

Recent studies have shown that some ER stress-inducible genes are involved in regulation of the UPR. For example, GRP78 binds to the luminal domain of UPR sensor proteins, PERK and IRE1 in addition to ATF6 and keeps them inactive in the ER (9, 12). Also, p58ipk, a HSP40 family protein, binds to the kinase domain of PERK and is capable of attenuating PERK-mediated translational repression (13). Likewise, GADD34, a regulatory subunit of protein phosphatase 1, functions as a negative feedback loop of the PERK pathway by facilitating dephosphorylation of phospho-Ser442 on the translational initiation factor eIF2-α (14). During the late phase of the UPR, unsimple
X-box-binding protein 1 (XBP1) is induced and forms a complex with active XBP1, a strong UPR transcription factor that is produced by IRE1-mediated unconventional splicing of XBP-1 mRNA (15). The complex formation leads to rapid degradation of both XBP-1 proteins (16). These findings raise the possibility that there are some genes that have the ability to modulate the UPR among uncharacterized ER stress-inducible genes.

Herein we focus on NUCB1, one of ER stress-inducible genes that we identified using a cDNA microarray system. NUCB1, also known as nucleobindin or calnuc, was first found as a secretory protein that possessed a signal sequence, a leucine zipper structure, two EF-hand motifs and a basic amino acid-rich region (17, 18, 19). Indeed, NUCB1 has been shown to be secreted in culture supernatant of a murine B cell line established from mice prone to the autoimmune disorder systemic lupus erythematosus (17, 20, 21). Interestingly, further studies have also demonstrated that NUCB1 is expressed ubiquitously and localizes in the Golgi apparatus of intact cells (19, 22). Although Golgi-localized NUCB1 has been suggested to be involved in Ca	extsuperscript{2+} homeostasis (23), the function of NUCB1 is largely unknown. In this study, we demonstrate that Golgi-localized NUCB1 suppresses ATF6 activation by inhibiting S1P-mediated cleavage during ER stress.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—Deoxyglucose (Sigma) and DTT (Nacalai Tesque; Kyoto, Japan) were dissolved in distilled, sterilized water. Tunicamycin (Nacalai Tesque), thapsigargin (Wako Pure Chemical Industries; Osaka, Japan), and MG132 (Peptide Institute, Inc.; Osaka, Japan) were dissolved in dimethyl sulfoxide. These compounds were added to culture medium with the solvent being less than 0.5% of the volume of the culture medium. Rabbit polyclonal anti-NUCB1 antibody was raised against synthetic peptide corresponding to C-terminal CEKKLERLPEVEPQH of the human NUCB1 protein. Rabbit polyclonal anti-ATF6 was prepared, as previously described (8). The following commercially available antibodies were used: mouse monoclonal anti-KDEL (StressGen; Victoria, BC, Canada), anti-calseamin, anti-ERp72 (BD Biosciences, San Jose, CA), anti-golgin97 (Invitrogen, San Diego, CA), anti-FLAG M2, anti-β-actin (Sigma), and HRP- or FITC-conjugated anti-V5 (Invitrogen), HRP-conjugated anti-rabbit or mouse IgG (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

**Cell Culture and Treatments**—Cells were maintained in RPMI 1640 medium (for HT1080) or Dulbecco’s modified Eagle’s medium (for 293T and HeLa) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin and were cultured at 37 °C in a humidified atmosphere containing 5% CO	extsubscript{2}. Glucose-free RPMI 1640 medium was obtained from Invitrogen and supplemented with 10% heat-inactivated fetal bovine serum for experimental use, as described (24). To induce UPR, cells underwent glucose deprivation for varying times. This was achieved by replacing the medium with the glucose-free medium, or chemical stressors 2DG, tunicamycin, thapsigardin, or DTT-containing medium (24). In some experiments, we treated the cells with the proteasome inhibitor MG132 at 10 μM during exposure to the UPR inducers.

**cDNA Microarray Analysis**—cDNA microarray analysis was performed as described previously (25, 26).

**RT-PCR Analysis**—RT-PCR was performed as previously described (24). Briefly, total RNA was isolated from cells using the RNeasy Mini Kit with DNase digestion (Qiagen, Tokyo, Japan) and converted to cDNA with SuperScript II reverse transcriptase (Invitrogen). The cDNAs for NUCB1, GRP78, and G3PDH were then amplified by PCR with PfuTurbo DNA polymerase (Stratagene). PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

**Immunoblot Analysis**—Cells were lysed in 1× SDS sample buffer, and protein concentrations of the lysates were measured with a Bio-Rad protein assay kit. For analysis of secreted proteins, culture medium (serum-free) was concentrated with Microcon YM-30 (Millipore; Billerica, MA) and then boiled in 1× SDS buffer. Equal amounts of proteins were resolved on a 10% SDS-polyacrylamide gel and transfected by electrophoretic transfer to a nitrocellulose membrane. Membranes were probed with antibodies, as indicated, and the specific signals were detected using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences Corp) (24).

**Immunoprecipitation**—Cells were washed with ice-cold PBS and lysed in 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA for NUCB1 or 50 mM Tris-HCl (pH 8.0), 1.5% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholic acid, 150 mM NaCl, 2 mM MgCl	extsubscript{2} for S1P (27) supplemented with a protease inhibitors mixture (Sigma). The lysates were cleared by centrifugation at 13,000 × g for 10 min at 4 °C and immunoprecipitated by anti-NUCB1 or anti-V5-conjugated beads (Sigma) in lysis buffer. Immunoprecipitates were washed three times with lysis buffer and eluted by boiling in SDS sample buffer for immunoblot analysis.

**Immunofluorescence**—Cells on a poly-lysine-coated coverslip were fixed and permeabilized for 10 min in PBS-containing 4% paraformaldehyde and 0.1% Triton X-100. After blocking for 1 h in PBS with 10% bovine serum albumin, the cells were incubated with primary (mouse anti-FLAG M2, 1:1000; rabbit anti-NUCB1, 1:3000; or mouse anti-golgin 97, 1:1000) and subsequent secondary antibodies (Alexa-fluor 488 conjugated anti-mouse IgG (1:1000), or Alexa-fluor 568-conjugated anti-rabbit Ig (1:1000); Invitrogen) in PBS with 1.5% bovine serum albumin for 1 h at room temperature. The coverslips were mounted on microscope slides and observed under a Nikon Diaphot 300 microscope at 400× magnification with appropriate filters to detect fluorescence.

**Plasmids**—pcFLAG vector, pATF6wt, pATF6act, pXBP1sp, pGRP78pro160-Luc have been described previously (24). The plasmid of 7× FLAG-tagged ATF6 was produced by tandem ligation of an oligonucleotide DNA sequence encoding 3× FLAG epitope to the HindIII site of pATF6wt. Expression vectors for non-tagged, V5-tagged and FLAG-tagged NUCB1 proteins were constructed by inserting each cDNA amplified by RT-PCR into pcDNA3 (Invitrogen), pcDNA3.1 TOPO V5/His (Invitrogen) and pFLAG-CMV-5c (Sigma), respectively. Expression vectors for IRE1 and S1P were constructed by inserting each cDNA amplified by RT-PCR (for IRE1) or by PCR using a KIAA0091 clone (kindly provided from Kazusa...
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DNA Research Institute (28) as a template (for S1P) into the pcDNA3.1 TOPO V5/His vector (Invitrogen). Reporter plasmids containing a promoter region of NUCB1 were generated by ligating products amplified by PCR, using genomic DNA from HT1080 cells as a template, into pGL3-basic vector (Promega). Site-directed mutagenesis was carried out using a QuikChange mutagenesis kit (Stratagene) for the NUCB1 mutant, P28A plasmid, which was exchanged Pro to Ala at position 28. The proper construction of plasmids was confirmed by DNA sequencing.

**Transfection**—Transient transfections were performed using either FuGENE6 Transfection Reagent (Roche Applied Sciences, Indianapolis, IN) for HT1080 cells or Lipofectamine 2000 (Invitrogen) for 293T, according to each manufacturer’s protocol.

**Reporter Assay**—HT1080 cells (2 x 10⁵ in each well of 12-well plates) were cultured overnight under normal growth conditions. After changing the medium to antibiotic-free RPMI 1640 medium supplemented with 5% fetal bovine serum, we incubated the cells for 6 h at 37 °C in a CO₂ incubator with transfection mixtures containing the indicated amounts of NUCB1 plasmids and 375 ng of the firefly luciferase-containing reporter plasmids pGRP78pro160-Luc with 125 ng of plasmid pRL-TK (Promega) (in which Renilla luciferase expression is under the control of the herpes simplex virus thymidine kinase promoter) as an internal control. The medium was then replaced with fresh medium lacking plasmids, and the cells were incubated under the same conditions for another 6 h. The cells were then treated for 12 h with UPR inducers. Relative firefly-to-Renilla luciferase activity (means with S.D. values of triplicate determinations) was determined using the dual luciferase kit (Promega). For NUCB1 promoter analysis, HT1080 cells (1 x 10⁵ in each well of 24-well plates) were transiently transfected with 500 ng of pNUCB1-Luc (firefly) plasmids, which contained different lengths (−584 to +112, −184 to +16, −134 to +16, −24 to +112) or point mutants of the NUCB1 promoter region (see Fig. 2) together with 1 ng of pRL-hCMV plasmid (Renilla) (Promega) as an internal control and then assayed as described above.

**RNA Interference**—Double-stranded RNA duplexes corresponding to human NUCB1 (1: 5′-AAGGTCTACAAGGGCTACGAGA-3′ and 2: 5′-TCAGTGATCGGCTAACAATTA-3′), as well as Control siRNA were purchased from Qiagen. For transient transfection of siRNA, cells were seeded at a density of 0.5 x 10⁵/well in a 12-well plate and were cultured overnight. The cells were transfected for 6 h with siRNA (80 nM), using Lipofectamine 2000 reagent according to the manufacturer’s protocol. Two days after transfection, the cells were used for experimentation.

**Sialidase Treatment**—NUCB1 proteins prepared from cells were immunoprecipitated with anti-NUCB1. After elution using synthetic peptide, each sample was digested with sialidase II (Clostridium perfringens) at 37 °C for 3 h.

**RESULTS**

**NUCB1, an ER Stress-responsive Gene**—We conducted a microarray analysis of gene expression to identify novel genes induced by glucose starvation, one of the physiological ER stress conditions. For this purpose, we used mRNAs from human ovarian cancer A2780 and colon cancer HT29 cells that had been cultured for 18 h under normal or glucose starvation conditions. As a result, NUCB1 was identified as a glucose starvation-inducible gene. To validate the finding, we analyzed mRNA expression of NUCB1 by RT-PCR in human fibrosarcoma HT1080 cells (Fig. 1A). Enhanced NUCB1 expression, as well as GRP78 expression, was seen in cells starved of glucose for 18 h. NUCB1 was also induced by treatment with 2-deoxyglucose (2DG) and tunicamycin, representative chemical ER stressors. Immunoblot analysis revealed that NUCB1 protein was also up-regulated by glucose withdrawal or 2DG treatment in HT1080 and HeLa cells (Fig. 1B).

As previously reported (19), immunostaining demonstrated that NUCB1 localized at the Golgi apparatus (see Fig. 5C). However, NUCB1 was also reported as a secretory protein in mouse pituitary AtT20 and normal rat kidney cells (21). To verify secretion of NUCB1, we cultured HT1080 cells in serum-free medium in the presence of 2DG or tunicamycin and then recovered the culture medium for immunoblot analysis. NUCB1 was detected in the culture medium under both normal and stress conditions, and the secretion was increased by the chemical stressors within 3 h (Fig. 1C). At that time point, GRP78 induction was marginal, suggesting that the increased NUCB1 secretion occurred at a relatively early phase of the UPR. These results indicate that NUCB1 can be Golgi-resident inside the cells and can be secreted outside the cells.

NUCB1 has been shown to be modified with sialylated O-linked oligosaccharides (21). To determine sialylated status of NUCB1, we immunopurified NUCB1 proteins and digested them with sialidase (Fig. 1D). The sialidase treatment resulted
in an approximate 1–2-kDa shift in extracellular pool mobility of NUCB1 proteins, indicating that secreted NUCB1 proteins were indeed sialylated. A similar mobility shift was seen in intracellular pools of NUCB1 proteins in both unstressed and 2DG-stressed cells, which had been detected as two bands and resulted in the disappearance of the upper band (Fig. 1D).

Together, these results suggested that sialylation might be an essential modification for secretion of NUCB1.

**ER Stress-responsive Elements in NUCB1 Promoter**—We cloned an approximate 700-bp putative promoter region of the human NUCB1 gene, including the transcriptional initiation site (−584 to +112), into a luciferase reporter vector (pNUCB1-Luc). The pNUCB1-Luc or the SV40 promoter-driven pGL3 (control) was transfected into HT1080 cells. Treating the cells with tunicamycin for 18 h led to activation of the NUCB1 promoter, but not the SV40 promoter, in a dose-dependent manner (Fig. 2A). We also found that co-transfection of pATF6act or pXBP1sp, the active forms of UPR transcription factors ATF6 and XBP1, respectively (8, 15), resulted in a strong activation of NUCB1 promoter activity (Fig. 2A).

**FIGURE 2. ER stress-responsive elements in NUCB1 promoter.** A, reporter gene including NUCB1 promoter region (left). Position +1 represents the transcriptional initiation site. HT1080 cells were transiently transfected with pNUCB1-Luc together with pRL-hCMV as the internal control. The cells were then treated with the indicated doses of tunicamycin for 16 h (middle) or were incubated in fresh medium for 18 h after co-transfection with pATF6act or pXBP1sp (right). Luciferase activity was measured with a dual luciferase assay kit. B, cis-elements in NUCB1 promoter. ERSE II and UPRE-like sequences were outlined (left), and the direction was indicated by arrow, respectively. Mutations introduced into ERSE II and UPRE-like (bold) are shown as enlarged characters in Mut-1, -2, and -3 (right). C, reporter assay using each of the pNUCB1-Luc (left) was performed as in A. D, HT1080 cells were transiently transfected with NUCB1-Luc1 or each of the constructs shown in B, and luciferase activity was determined as above. Each activity of firefly or Renilla luciferase is shown separately in supplemental Fig. S1, A, B, and C.
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Examination of the NUCB1 promoter region revealed one ERSE II (−124～−114, inverted) and one unfolded protein response element (UPRE)-like sequence (−152～−144) as potential ER stress-responsive cis-elements (Fig. 2B). We tested the role of the regions in promoter activity using pNUCB1-Luc1 (−184～+7), a deletion version of pNUCB1-Luc that still contains both the UPRE-like and the ERSE II regions. The pNUCB1-Luc1 was strongly activated by tunicamycin treatment (18 h) and by co-transfection with pATF6act or pXBP1sp (Fig. 2C). Deletion of the UPRE-like region (pNUCB1-Luc2: −134～+16) decreased the promoter activation without affecting the basal promoter activity under normal conditions. Further bulky deletion (pNUCB1-Luc3: −24～+112) led to promoter activation loss and remarkable decrease in the basal promoter activity. Consistently, each mutation of UPRE-like (Mut1) and ERSE II (Mut2 and 3) in pNUCB1-Luc1 decreased promoter activation by tunicamycin treatment and by pATF6act or pXBP1sp co-transfection (Fig. 2D). Some variations were seen in the inhibitory effect of each mutation, depending on the promoter activation conditions. Combined mutation of both elements resulted in further promoter activation decrease. Interestingly, the point mutations had a marginal effect on the basal promoter activity. These results demonstrated that both ERSE II and UPRE-like regions mediate NUCB1 promoter activation during ER stress.

NUCB1 Represses UPR with Inhibiting ATF6 Activation—To examine the role of NUCB1 in the UPR, we used pGRP78-Luc, which contained a GRP78 promoter region (−160～+7) immediately upstream of the firefly luciferase. As previously reported (3), the GRP78 promoter reporter was activated by treating the transfected cells with 2DG or tunicamycin in a dose-dependent manner (Fig. 3A). We found that co-transfection of NUCB1 significantly attenuated the GRP78 promoter activity induced by each stressor (Fig. 3A). Consistently, immunoblot analysis of lysates from 293T cells revealed that NUCB1 overexpression significantly attenuated induction of endogenous UPR target proteins under stress conditions (Fig. 3B).

GRP78 promoter activity was also enhanced in proportion to the transfected amount of pATF6wt (Fig. 4A, left). Under the experimental conditions, the ATF6wt-induced GRP78 promoter activation was decreased remarkably by co-transfection of NUCB1. In contrast, NUCB1 had little effect on the promoter activity induced by the active form of ATF6 (Fig. 4A, middle). As to the IRE1-XBP1 pathway, NUCB1 did not affect IRE1- or XBP1sp-mediated activation of the GRP78 promoter (Fig. 4A, right).

We next examined the effects of NUCB1 on the activation process of ATF6. For this purpose, we used a construct of 7×FLAG-tagged ATF6 to increase the detection sensitivity to the small amount of ATF6 because extremely overexpressed ATF6 can be readily activated by proteolytic cleavage independently of ER stress (11). When the plasmid of 7×FLAG-tagged ATF6 was transfected into HT1080 cells, the ATF6 activation process was detected in an ER stress-dependent manner (Fig. 4B). Indeed, in addition to p90ATF6, an intermediate product (S1P cleavage product) and p50ATF6/active form (sequential S2P cleavage product) emerged after thapsigargin treatment. We found that the production of processed ATF6 was decreased by co-transfection of NUCB1 (Fig. 4B, left). Conversely, siRNA-mediated NUCB1 knockdown enhanced the processed form production during tunicamycin or thapsigargin treatment (Fig. 4B, right).

We also examined the effects of NUCB1 overexpression on endogenous ATF6 processing (Fig. 4C, left). When 293T cells were treated with tunicamycin for 8 ～12 h, p50ATF6 as well as the underglycosylated form of p90ATF6 were produced. The production of p50ATF6 was attenuated by overexpression of NUCB1. In agreement with this finding, silencing of NUCB1 in HT1080 cells enhanced the production of endogenous p50ATF6 during tunicamycin treatment for 8 or 12 h, as compared with non-silencing cells (Fig. 4C, right and supplemental Fig. S5). These results indicate that NUCB1 can negatively regulate ATF6 processing for its activation during ER stress.

NUCB1 Represses ATF6 Activation in the Golgi Apparatus—We examined whether or not NUCB1 overexpression influenced the subcellular localization of ATF6 (7×FLAG tag) (Fig. 5A). At 20 min after DTT addition, ATF6 was concentrated in Golgi-like structures either with or without NUCB1 overexpression (Fig. 5A, arrow). At 40 min, some populations of the cells transfected with pATF6 alone took up nucleus staining (Fig. 5A, arrowhead) while cells co-transfected with ATF6 and
NUCB1 suppresses ATF6 processing in the Golgi apparatus. Although the mechanism of ER localization of this mutant is largely unknown. We present evidence that NUCB1 can function as an ER stress-responsive negative regulator of ATF6 activation.

We compared the effects of P28A and WT on ATF6 processing. HT1080 cells were co-transfected with pATF6wt (7× FLAG-tag) and WT or P28A and then treated with thapsigargin (Fig. 5D). We found that P28A was scarcely able to repress thapsigargin-induced ATF6 processing (Fig. 5D). Consistently, P28A did not suppress nuclear translocation of ATF6, while, for unknown reasons, P28A stimulated it (Fig. 5E). Together, the Golgi localization of NUCB1 is likely required for inhibition of ATF6 processing.

**Impairment of Interaction between ATF6 and S1P by NUCB1**—We found that interaction between ATF6 and S1P was detected during ER stress by a co-immunoprecipitation assay. Indeed, co-transfection of pS1P (V5 tag) and pATF6wt (7× FLAG tag) made it possible to detect the interaction between ATF6 and S1P under conditions of 2DG, tunicamycin, and thapsigargin (Fig. 6A). We then examined the effects of NUCB1 on the stress-induced interaction between ATF6 and S1P. As shown in Fig. 6B, thapsigargin-induced interaction between ATF6 and S1P was clearly diminished by NUCB1 overexpression. At the same time, NUCB1 overexpression inhibited production of the active form of ATF6. Thus, NUCB1 prevented the interaction between ATF6 and S1P leading to ATF6 cleavage during ER stress.

In agreement with these findings, we also found that overexpression of S1P dose-dependently suppressed the inhibitory activity of NUCB1 against ATF6-mediated GRP78 promoter activation (Fig. 6C). Together, these results support the notion that the interaction between ATF6 and S1P is an important target for inhibition of ATF6 activation by NUCB1.

**DISCUSSION**

The ATF6 activation system must contain a negative feedback regulation mechanism because ATF6 processing was temporarily increased and then gradually attenuated during ER stress (3, 29). However, regulatory proteins for the process are largely unknown. We present evidence that NUCB1 can function as an ER stress-responsive negative regulator of ATF6 activation. Indeed, both mRNA and protein of NUCB1 are induced during ER stress. The promoter region of the NUCB1 gene con-
NUCB1 protein localizes in the Golgi apparatus, the intracellular site where ATF6 is cleaved by S1P/S2P for nuclear translocation. In agreement with intracellular localization, NUCB1 suppresses physical interaction of S1P-ATF6 and subsequent S1P-mediated cleavage of ATF6 during ER stress. Collectively, these findings demonstrate that NUCB1 can be involved in a negative feedback loop of the ATF6-mediated branch of the UPR.

We showed herein that ATF6 activation is suppressed by overexpression of NUCB1, whereas it is enhanced by knockdown of NUCB1. Thus, the increased expression of NUCB1 during ER stress can be an important mechanism to suppress ATF6 activation. As observed for NUCB1, certain UPR-inducible genes have also been reported to exhibit UPR-modulating activity (9, 13, 14, 16). As to ATF6 regulation, GRP78, a major UPR target chaperone protein, has been shown to down-regulate the ATF6 activation process by preventing transport of newly synthesized ATF6 from the ER to the Golgi apparatus (9). Thus, the ATF6 activation process can be regulated by GRP78 in the ER and by NUCB1 in the Golgi. In addition to expression levels, subcellular localization of NUCB1 may contribute to ATF6 regulation during ER stress. Indeed, we observed a tendency that increased secretion of NUCB1 occurred sooner than increased intracellular expression of NUCB1 during ER stress (Fig. 1C).

While previous studies demonstrated that extracellular NUCB1 can stimulate B cell growth (20), we observed no effect of silencing NUCB1 on HT1080 fibrosarcoma cell growth (data not shown), which suggests that the growth-promoting activity of NUCB1 may be cell type-dependent. Alternatively, the secreted form may contribute to modulating the stressed cell microenvironment. Actually, extracellular NUCB1 has been suggested to serve as a modulator of matrix maturation in bone, based on the observations that NUCB1 is secreted by osteoblasts and osteocytes and can indeed be detected in the osteoid extracellular matrix (18, 30). Thus, NUCB1 secretion might have a role not only in facilitating the ATF6 activation process inside stressed cells but also in modulating stressful microenvironment outside the cells.

We showed that as an inhibition mechanism of ATF6 activation, NUCB1 prevented S1P-ATF6 interaction during ER stress. However, we were unable to detect a binding between NUCB1 and either ATF6 or S1P under the same experimental conditions that the S1P-ATF6 interaction was easily seen. This
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may have been the result of technical problems because we could detect an interaction between NUCB1 and ATF6 when both proteins were extremely overexpressed by transfection (data not shown). Thus, a complex between NUCB1 and ATF6 or S1P may be too sensitive to detergents or too transient to be detected under our experimental conditions. We speculate that NUCB1 requires cooperating protein(s) to bind to ATF6 or S1P and to prevent S1P-ATF6 interaction. In other words, the S1P-ATF6 interaction during ER stress may be an event regulated by several factors containing NUCB1 but not be a mere consequence of ATF6 translocation from ER to the Golgi apparatus. In this context, it may be worth noting that NUCB1 has been reported to be able to interact physically with other proteins, such as cyclooxygenase I and a G protein, Gsig3 (31, 32). It would be interesting to examine whether such proteins are involved in regulation of the UPR, especially the ATF6-mediated branch.

In conclusion, our studies identified NUCB1 as a novel UPR negative regulatory protein that suppresses the S1P-mediated cleavage activation of ATF6 in the Golgi apparatus. In addition to ATF6, there are several members of ER-anchored transcriptional factors that are structurally similar to ATF6 (33, 34, 35). These members, including SREBP2, OASIS and CREBH, all have been shown to be activated by S1P/S2P-mediated cleavage, the so-called, RIP. However, it is unlikely that RIP is generally regulated by NUCB1 because overexpression or siRNA-mediated knockdown of NUCB1 had little effect on SREBP2 processing (data not shown). Thus, these findings provide a clue to elucidating regulation mechanisms of RIP for each transcriptional factor at the Golgi apparatus. Besides, the finding of enhanced NUCB1 secretion during ER stress may also be intriguing, as increased NUCB1 secretion has reportedly been associated with induction or enhancement of autoimmunity in murine models of lupus (20). Thus, our findings could offer information for studying the relationship between the UPR and autoimmune response, as well as other UPR-associated pathophysiological states, such as tumor development, neurodegenerative disorders, and diabetes.

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