TFE3 Is a bHLH-ZIP-type Transcription Factor that Regulates the Mammalian Golgi Stress Response

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ABSTRACT. The Golgi stress response is a mechanism by which, under conditions of insufficient Golgi function (Golgi stress), the transcription of Golgi-related genes is upregulated through an enhancer, the Golgi apparatus stress response element (GASE), in order to maintain homeostasis in the Golgi. The molecular mechanisms associated with GASE remain to be clarified. Here, we identified TFE3 as a GASE-binding transcription factor. TFE3 was phosphorylated and retained in the cytoplasm in normal growth conditions, whereas it was dephosphorylated, translocated to the nucleus and activated Golgi-related genes through GASE under conditions of Golgi stress, e.g. in response to inhibition of oligosaccharide processing in the Golgi apparatus. From these observations, we concluded that the TFE3-GASE pathway is one of the regulatory pathways of the mammalian Golgi stress response, which regulates the expression of glycosylation-related proteins in response to insufficiency of glycosylation in the Golgi apparatus.

Key words: Golgi stress, proteoglycan, sialyltransferase, glycosylation, ER stress

Introduction

The endoplasmic reticulum (ER) and the Golgi apparatus are organelles involved in the synthesis and maturation of secretory proteins, respectively. In the ER, secretory proteins are synthesized and folded with the assistance of ER chaperones, while misfolded proteins are degraded by a process known as ER-associated degradation (ERAD) (Hampton, 2002). When the synthesis of secretory proteins increases and overwhelms the capacity of ER chaperones and ERAD, unfolded proteins accumulate in the lumen of the ER, which could lead to apoptotic cell death. To cope with such an insufficiency of ER function (ER stress), eukaryotic cells activate homeostatic mechanisms called the ER stress response (also called the unfolded protein response) and increase the protein-folding capacity of the ER (Kimata and Kohno, 2011; Mori, 2009; Ron and Harding, 2012; Walter and Ron, 2011; Wang and Kaufman, 2012; Yoshida, 2009). The mammalian ER stress response consists of three main mechanisms: the ATF6, IRE1 and PERK pathways. Sensor molecules in each pathway detect unfolded proteins accumulating in the ER and upregulate the transcription of ER-related genes encoding ER chaperones and ERAD components, resulting in the augmentation of ER function.

When the capacity of the ER is increased by the ER stress response and a large amount of secretory proteins are transported from the ER to the Golgi apparatus, the Golgi becomes inundated with an excess of secretory proteins. It was suggested that eukaryotic cells must have a homeostatic mechanism, similar to the ER stress response, which induces the transcription of Golgi-related genes to cope with such an insufficiency of Golgi function (Golgi stress). We named this homeostatic mechanism the Golgi stress response.

In a previous report, we found that the expression levels
of Golgi-related genes encoding glycosylation enzymes (sialyltransferase 4A (SIAT4A), sialyltransferase 10 (SIAT10), fucosyltransferase 1 (FUT1) and UDP-N-acetylhexosamine pyrophosphorylase-like 1 (UAP1L1)), a Golgi-structural protein (GCP60) and components of vesicular transport (syntaxin 3A, RAB20, WIP149, Giantin and GM130) (Band and Kuismann, 2005; Jeffries et al., 2004; Kitagawa and Paulson, 1994; Lutcke et al., 1994; Mio et al., 1998; Mollicone et al., 1994; Munro, 2011; Sohda et al., 2001; Taniguchi et al., 2001) were increased during Golgi stress (Oku et al., 2011). Analysis of the promoters of these genes revealed that their transcriptional induction in response to Golgi stress is commonly regulated by a novel enhancer element called the Golgi apparatus stress response element (GASE), whose consensus sequence is ACGTGGC. These findings were the first evidence indicating the existence of a Golgi stress response, although the mechanism of the Golgi stress response still remains unknown.

Here, we isolated a transcription factor, TFE3, which binds to GASE and activates the transcription of Golgi-related genes, and we analyzed the activation mechanism of TFE3 in response to Golgi stress. We also examined what molecular changes in the Golgi apparatus induce the Golgi stress response. Our analysis revealed the core regulatory mechanism of the Golgi stress response in mammalian cells.

Materials and Methods

Cell culture and transfection

Culture of HeLa, L and sog9 cells was carried out as described previously (Nadanaka et al., 2008; Uemura et al., 2009). Transfection of plasmid DNAs was performed by either the calcium phosphate method or lipofection using FuGene (Roche, Basel, Switzerland). After transfection, cells were treated with Golgi stress inducers for 12–16 h, washed three times with PBS and harvested for immunoblotting and immunocytochemistry (Komori et al., 2012). For luciferase assays, transfected cells were incubated in fresh medium for an additional 6 h for recovery before harvest (Oku et al., 2011). Separation of the nuclear and cytoplasmic fractions was carried out as described previously (Schreiber et al., 1989).

Construction of plasmids and transfection of siRNAs

Plasmids expressing human TFE3 were constructed by inserting cDNAs containing the entire coding regions into vector pcDNA3.1 (Invitrogen, CA). Point mutants were constructed by site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene, CA). To construct shRNA-expressing plasmids, the following regions of the TFE3 cDNA were inserted into the BglII site of an expression plasmid pSuper (Oligoengine, WA): shTFE3-A (GATCCCCGGAGTCCAGTTAACATGATGACGAATCATGAACTGGACTCCTTTTTTA) and shTFE3-B (GATCCCCGGAGTCCAGTTAACATGATGACGAATCATGAACTGGACTCCTTTTTTA). The nucleotide sequences of siRNAs used in this study were as follows: siTFE3-A (CAGAAAGAGAAACCAACACCUA), siTFE3-B (GGGAUACUGCUUAGUGUAACAGUA), siSLC35A1-A (UAGGUAUAGACUGACGACAT), siSLC35A-B (GGGAUACCGAACCAATT), si-mTOR-A (UGUUAUCCCCAACACGAGCag), si-mTOR-B (UAUGGACUGAAUGCAGAUGt) and si-mTOR-C (UAAGGAUCACAAAGGCUCt). Silencer Select Negative Control #4390843 (Life Technologies, CA) was used as the control siRNA. Transfection of siRNA was carried out using a lipofection agent, RNAi MAX (Life Technologies), and transfected cells were incubated for 48 h after transfection.

Preparation of anti-TFE3 antisera

Anti-TFE3-A antiserum was purchased from Abcam (ab93808; Cambridge, UK). In addition, a fusion protein between a region [441-575] of human TFE3 and bacterial glutathione S-transferase (GST) was expressed in Escherichia coli and used as an antigen for preparation of polyclonal anti-TFE3-B and anti-TFE3-C antisera in rabbits. The resulting antisera were purified twice through a column conjugated with GST and bacterial lysates and an affinity column conjugated with antigen.

Northern blotting, immunoblotting and immunocytochemistry

Northern blotting, immunoblotting and immunocytochemistry were carried out as described previously (Yoshida et al., 2001). The cDNA probe used for Northern blotting corresponded to region [1001-1500] of the TFE3 mRNA. Anti-ATF6 and anti-XBP1 antisera were purchased from Cosmo Bio (Tokyo, Japan) (clone 1–7) and Santa Cruz Biotechnology (TX) (sc-7160), respectively.

Disaccharide analysis of heparan sulfates

Heparan sulfates (HSs) were isolated and purified from HeLa cells as described previously (Nadanaka et al., 2008; Nadanaka and Kitagawa, 2008). Purified HSs were digested with a mixture of heparinase from Flavobacterium heparinum (EC 4.2.2.7) (1 mIU) and heparitinase from Flavobacterium heparinum (EC 4.2.2.8) (1 mIU) at 37°C for 4 h. The digested products were derivatized with fluorophore 2-aminobenzamide and then analyzed by high-performance liquid chromatography (HPLC) as reported previously (Nadanaka et al., 2011, 1998; Okada et al., 2010).

Chromatin immunoprecipitation assay

Genomic DNA and proteins in whole cell lysates were cross-linked using 11.1% formaldehyde, and genomic DNA was disrupt-
ed by sonication using a Bioruptor (Cosmo Bio), and TFE3-DNA complexes were immunoprecipitated using anti-TFE3-A antiserum (Nakagawa et al., 2006). GASE or ER stress response element (ERSE) sequences were amplified by PCR using co-precipitated genomic DNA as a template and separated on a TAE-2% agarose gel.

**Quantitative RT-PCR and luciferase assays**

Quantitative RT-PCR (qRT-PCR) was carried out using an ABI 7500 qPCR instrument (Life Technologies) and a PrimeScript RT reagent kit with gDNA Eraser and SYBR Premix Ex Taq II (Tli RNase H Plus) (TaKaRa, Otsu, Japan). Primer pairs used for qRT-PCR were as follows: GCP60 (AGCGTGACATGGCATGAGCTTCCATCCAA and GCCACAATCTCATCCAGCAAG), SIAT4A (ATGCATTGAGCTTGGGTCAGG and CCAGCTTGATGAAAGCAGAAG), SIAT10 (GAGCTTGGGACAGCTTCTGTTG and ACTCAGGAAATATGGCCACAAGATAC), UAP1L1 (GTGGTGAGTACCCAGGGCAAAGGTG). Assays of firefly luciferase were performed using a PicaGene Dual Sea Pansy luminescence kit (Toyo Ink, Tokyo, Japan).

**Results**

**bHLH-ZIP transcription factor TFE3 activates transcription of Golgi-related genes through the specific GASE enhancer element**

Recently, we identified the novel enhancer element GASE, which regulates Golgi stress-induced transcription (Oku et al., 2011), and isolated a transcription factor MLX as a GASE-binding protein using a yeast one-hybrid screen (Taniguchi et al., manuscript in preparation). Since knockdown of MLX reduced GASE-mediated transcriptional induction but its overexpression hardly enhanced it, it seems that MLX is not the main transcription factor that regulates the mammalian Golgi stress response. Because MLX is a bHLH-ZIP protein, it was likely that other bHLH-ZIP type transcription factors may be involved in GASE-mediated transcriptional induction upon Golgi stress. Among 28 bHLH-ZIP proteins encoded in the human genome, we noticed that the consensus DNA sequences to which the bHLH-ZIP transcription factor TFE3 binds (μE3 and USF sites, with consensus sequences of CAGGTGGC and CACGTGGC, respectively) (Beckmann et al., 1990; Lenardo et al., 1987) were very similar to the GASE sequence (Fig. 1A). Therefore, we first examined whether TFE3 can bind to GASE using a chromatin immunoprecipitation (ChIP) assay (Fig. 1B). Endogenous TFE3 contained in cell lysates prepared from HeLa cells treated with or without the Golgi stress-inducing reagent monensin (Oku et al., 2011) (see Discussion) was immunoprecipitated with anti-TFE3-A antiserum, and co-precipitated genomic DNA was used as a template for amplification of the DNA fragment containing the GASE sequence of a human SIAT4A or GCP60 promoter. In control cells, the promoter regions of the human SIAT4A or GCP60 genes, including a GASE sequence, was hardly amplified (upper and middle panels, lanes 3), whereas the corresponding region was specifically amplified when cells were treated with monensin (lane 4). In contrast, an ERSE sequence of the human BiP promoter, which is responsible for ER stress-induced transcription (Yoshida et al., 1998), was hardly amplified in either monensin-treated or untreated cells (lower panel, lanes 3 and 4). These findings suggested that the binding of TFE3 to GASE sequences is increased in response to Golgi stress.

Next, we examined whether TFE3 can activate GASE-mediated transcription. When overexpressed in HeLa cells, TFE3 increased the transcription of a reporter plasmid containing a firefly luciferase gene under the control of a GASE enhancer (GASE-LUC) by more than 20-fold (Oku et al., 2011) (Fig. 1C, lanes 1 and 2), whereas it hardly affected transcription from a GASE-mutant reporter (lanes 3 and 4), suggesting that TFE3 can specifically activate GASE-mediated transcription.

We also examined whether the suppression of TFE3 expression affected Golgi stress-induced transcription. First, suppression efficiency by shRNA and siRNA was evaluated. Endogenous TFE3 protein was detected as two sets of four bands by immunoblotting using anti-TFE3-A antiserum (Fig. 1D, upper panel, lane 1), and we designated these larger and smaller sets TFE3(L)-0 to -3 and TFE3(S)-0 to -3, respectively (also see Fig. 2B, lane 1). The number represents the amount of the phosphorylation in TFE3 (“0” means non-phosphorylated and “3” means highly phosphorylated). We tested three independently prepared antisera against TFE3 and obtained identical results (Fig. S1B, lanes 1 and 4). We also checked whether anti-TFE3 antisera specifically recognized TFE3 (Fig. S1A). TFE3-myc as well as other closely related bHLH-ZIP proteins, such as TFEB-myc, MITF-myc and MLX-myc, were overexpressed in HeLa cells and subjected to immunoblotting. None of the three anti-TFE3 antisera cross-reacted with these bHLH-ZIP proteins (lanes 8–10, 13–15 and 18–20), but all three specifically detected TFE3 protein (lanes 7, 12 and 17). Expression of these dHLH-ZIP proteins was confirmed with the use of an anti-myc antibody (lanes 2–5). As for suppression efficiency, expression of TFE3 was hardly affected by a control shRNA (shControl) (Fig. 1D, upper and lower panels, lane 1), but suppressed to 15.2% and 8.7% by shRNAs for TFE3 (shTFE3-A and -B) (lanes 2 and 3). Expression of β-tubulin was not changed by either
Fig. 1. TFE3 is important for Golgi stress-induced transcription and Golgi function. (A) Comparison between the GASE sequence found in the human SIAT4A promoter and the consensus sequences of μE3 and USF. Identical nucleotides are highlighted. (B) GASE-binding of TFE3 in HeLa cells was evaluated using ChIP assays. HeLa cells were treated with 5 μM monensin for 4 h and TFE3-genomic DNA complex was immunoprecipitated using anti-TFE3-A antiserum. Co-immunoprecipitated GASE or ERSE sequences were amplified by PCR, and separated on a TAE-2% agarose gel. (C) Effect of TFE3 overexpression on GASE-mediated transcription. HeLa cells were transiently transfected with the indicated plasmids and subjected to luciferase assays. The activity in lane 1 is set as 1. (D and E) Confirmation of suppression of TFE3 expression by shRNA (D) and siRNA (E). HeLa cells transiently transfected with shRNA or siRNA for TFE3 (shTFE3-A, shTFE3-B, siTFE3-A or siTFE3-B) or control scrambled shRNA or siRNA (shControl or siControl) were subjected to immunoblotting with anti-TFE3-A (upper panel) or anti-β actin anti-serum (middle panel). Quantitation of TFE3 expression is shown in the lower panel. (F) Effect of TFE3 knockdown on transcriptional induction from GASE. TFE3-knockdown cells were transfected with a GASE-LUC reporter and subjected to luciferase assays after treatment of 0.3 μM monensin for 16 h. Fold induction after monensin treatment is shown. (G) Effect of TFE3 knockdown on transcriptional induction of target genes of the Golgi stress response. HeLa cells were transfected with siTFE3-A, treated with 0.3 μM monensin and subjected to qRT-PCR with the indicated primers complementary to target genes. The expression of mRNA in lanes 1, 5, 9, 13, 17, 21 and 25 is set as 1. (H) Effect of TFE3 knockdown on proteoglycan glycosylation in the Golgi apparatus. Cellular heparan sulfate disaccharides were prepared from HeLa cells treated with siControl, siTFE3-A or siTFE3-B and measured by HPLC. The amount of heparan sulfate in lanes 1 and 3 is set as 1.
Fig. 2. TFE3 is dephosphorylated and changes its subcellular localization upon Golgi stress. (A) Molecular weight change of TFE3 during Golgi stress. HeLa cells were pre-treated with 40 μg/ml cycloheximide for 2 h, further treated with 5 μM monensin for 4 h and subjected to immunoblotting using anti-TFE3-A antiserum. Cells in lane 1 overexpressed TFE3, and the amount of protein loaded in lane 1 was one tenth of that loaded in the other lanes. Arrows indicate the positions of TFE3(L) and TFE3(S). (B) In vitro dephosphorylation of TFE3. Whole cell lysates prepared from HeLa cells were treated with calf intestinal alkaline phosphatase in vitro and subjected to immunoblotting using anti-TFE3-A antiserum. (C) Phosphorylation status and subcellular localization of TFE3 during Golgi stress. HeLa cells treated with 5 μM monensin for 4 h were separated into the nuclear and cytoplasmic fractions and subjected to immunoblotting with anti-TFE3-A (upper panel), anti-lamin A+C (middle panel) and anti-β-tubulin (lower panel) antisera. (D) Subcellular localization of TFE3 during Golgi stress. HeLa cells treated with 5 μM monensin for 3 h were subjected to immunocytochemistry using anti-TFE3-A antiserum. Bars, 10 μm. (E) Time course of dephosphorylation and nuclear translocation of TFE3 during Golgi stress. HeLa cells treated with 5 μM monensin for the indicated time periods were separated into cytoplasmic (Cy) and nuclear (Nu) fractions, and subjected to immunoblotting as in Fig. 2C. “W” indicates whole cell lysates. (F) Nuclear translocation of TFE3 during Golgi stress. HeLa cells treated with 5 μM monensin for the indicated time periods were subjected to immunocytochemistry using anti-TFE3-A antiserum. Fractions of cells in which TFE3 was localized in the nucleus were counted manually. One hundred cells were counted in each experiment and experiments were repeated two times.
shRNAs (middle panel, lanes 1–3). When siRNAs specific to the human TFE3 mRNA (siTFE3-A and -B) were introduced into HeLa cells, expression of TFE3 was reduced to 6.4% and 4.3% respectively (Fig. 1E, upper and lower panels, lanes 2 and 3), whereas that of β-tubulin was hardly affected (middle panel). These findings indicated that the suppression efficiency of shRNAs and siTFEs was adequate.

Next, we examined the effect of TFE3 suppression on GASE-mediated transcription. When an expression plasmid for shControl was co-transfected into HeLa cells with a GASE-LUC reporter plasmid, luciferase activity was increased in response to monensin treatment (Oku et al., 2011) (Fig. 1F, lane 1), indicating that GASE-mediated transcription was induced by Golgi stress in the presence of control shRNA. In contrast, co-expression of TFE3 shRNAs almost abolished transcriptional induction through GASE upon Golgi stress (lanes 2 and 3). TFE3 shRNAs hardly affected transcription from the mutant GASE (lanes 5 and 6). These results suggested that TFE3 is important for transcriptional induction through GASE in response to Golgi stress. We confirmed this notion using another Golgi stress inducer, xyloside, as described below (Fig. 5E).

We also examined transcriptional induction of target genes of the Golgi stress response in TFE3-knockdown cells by quantitative real-time PCR (qRT-PCR), using siTFE3-A (Fig. 1G) and siTFE3-B (Fig. S1C). Transcription of the target genes, including GCP60, SIAT4A, SIAT10, UAP1L1, Giantin, GM130 and FUT1, was induced by monensin treatment (lanes 2, 6, 10, 14, 18, 22 and 26), whereas this transcriptional induction was largely suppressed in TFE3-knockdown cells (lanes 4, 8, 12, 16, 20, 24 and 28). These results suggested that TFE3 is involved in the transcriptional induction of these Golgi-related genes upon Golgi stress.

Because TFE3 was responsible for the transcriptional induction of genes involved in glycosylation, it is possible that the capacity of glycosylation in the Golgi apparatus was reduced in TFE3-knockdown cells. To investigate this possibility, we measured the amount of heparin sulfate disaccharides (HS) contained in proteoglycans, and found that the content of HS was reduced in TFE3-knockdown cells as compared with control cells (Fig. 1H, lanes 2 and 4). This suggested that TFE3 is important for glycosylation in the Golgi apparatus. In the above experiments, synthesis of HS was suppressed by siTFE3 even though cells were not treated with monensin, suggesting that TFE3 is slightly activated by basal Golgi stress in normal growing cells due to the synthesis of secretory proteins, which is responsible for the transcriptional induction of genes involved in HS synthesis. Actually, TFE3 was slightly activated in normally growing cells (Fig. 2A, lane 2), suggesting the small activation of the Golgi stress response.

**TFE3 is dephosphorylated upon Golgi stress**

To investigate whether the expression of TFE3 is changed during Golgi stress, whole cell lysates prepared from HeLa cells treated with monensin were subjected to immunoblotting using anti-TFE3 antiserum (Fig. 2A). In the absence of monensin, endogenous TFE3 protein was detected as two sets of four bands (TFE3-0 to 3) (lane 2). When TFE3 was overexpressed from an expression vector containing the TFE3 cDNA, the expression levels of TFE3(L) and TFE3(S) were increased simultaneously (lane 1), suggesting that these two isoforms are not products derived from alternative splicing of the TFE3 mRNA but post-translationally modified products. It is possible that the N- or C-terminus of TFE3(L) is cleaved or TFE3(S) is conjugated with small molecules such as SUMO. Because these two set of four bands behaved similarly during Golgi stress (see below), we assumed that TFE3(L) and TFE3(S) have similar activity for transcriptional induction. After monensin treatment, two sets of four bands of TFE3 (TFE3-0 to -3) became two sets of two smaller bands (TFE3-0 and TFE3-1) (lane 4). We observed this molecular weight shift using other anti-TFE3 antisera (Fig. S1B). This molecular weight shift after monensin treatment was still observed even if the cells were treated with cycloheximide, which blocks de novo translation (Fig. 2A, lanes 3 and 5), suggesting that the molecular weight shift is caused by post-translational modification. Northern blotting analysis using total RNA extracted from cells treated with monensin revealed that the size and amount of TFE3 mRNA did not change after monensin treatment (Fig. S2A), whereas RT-PCR and sequencing analysis were unable to detect alternative splicing of the TFE3 mRNA (data not shown). These findings confirmed that the molecular weight shift of TFE3 during Golgi stress was caused by post-translational modification. When whole cell lysates prepared from monensin-untreated HeLa cells were treated with calf intestine alkaline phosphatase (TaKaRa Bio, Otsu, Japan) in vitro, the two sets of four bands of TFE3 (TFE3-0 to 3) reduced to the two smallest bands (TFE3-0 and TFE3(S)-0) in each set (Fig. 2B, lane 2). This indicated that TFE3 is multiply phosphorylated in normal growth conditions and dephosphorylated in response to Golgi stress.

It is also possible that TFE3 is not dephosphorylated in response to Golgi stress, but dephosphorylated TFE3 that is rapidly degraded in normal growth conditions is stabilized upon Golgi stress. However, we found that dephosphorylated TFE3 appeared upon monensin treatment even if de novo translation was blocked by cycloheximide (Fig. 2C, lanes 2–4), suggesting that dephosphorylated TFE3 emerging after monensin treatment was derived from pre-existing phosphorylated TFE3. In addition, 2 h of cycloheximide treatment hardly affected the expression level of either dephosphorylated or phosphorylated TFE3 (Fig. 2A, lane 3), indicating that TFE3 is not an especially unstable pro-
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tein and there is no difference in stability between phosphorylated and dephosphorylated TFE3. Moreover, we examined whether the proteasome is involved in degradation of TFE3 (Fig. S2B). MG132 treatment slightly increased the accumulation of dephosphorylated TFE3 (lane 4), although the level was much smaller than that observed after monensin treatment (lanes 2 and 3), suggesting that dephosphorylation of phosphorylated TFE3 upon Golgi stress is the major activation mechanism of TFE3, whereas stabilization of dephosphorylated TFE3 upon Golgi stress is the minor mechanism.

**TFE3 translocates from the cytoplasm to the nucleus upon Golgi stress**

Next, we examined the biological consequence of Golgi stress-induced dephosphorylation of TFE3. When plasma membranes from HeLa cells were mildly solubilized using the detergent NP-40 without disrupting the nuclear membrane, and the cytoplasm and the nucleus were fractionated, most of multi-phosphorylated TFE3 (TFE3-2 and TFE3-3) was localized in the cytoplasmic fraction (Fig. 2C, lane 3 and Fig. S2D), whereas less phosphorylated TFE3 (TFE3-0 and TFE3-1) was concentrated in the nuclear fraction (Fig. 2C, lane 1 and Fig. S2D). Upon Golgi stress, most TFE3 proteins became less phosphorylated and were found in the nuclear fraction (Fig. 2C, lane 2 and Fig. S2D). These observations suggested that TFE3 is multi-phosphorylated and anchored in the cytoplasm in normal growth conditions, whereas TFE3 is dephosphorylated, liberated from the cytoplasm and translocated into the nucleus upon Golgi stress, to upregulate GASE-mediated transcription.

We confirmed this notion by immunofluorescence microscopy. When endogenous TFE3 was detected using anti-TFE3-A antiserum in untreated HeLa cells, TFE3 was found in the cytoplasm as well as the nucleus (Fig. 2D, panels a–c). Upon monensin treatment, TFE3 became concentrated in the nucleus (panels d–f). We confirmed this finding using a C-terminally myc-tagged TFE3 construct (TFE3-myc) (Fig. S2E). When expressed in HeLa cells, TFE3-myc was detected in the cytoplasm in the absence of monensin (panels a–c), whereas it was concentrated in the nucleus upon monensin treatment (panels d–f). Nuclear translocation of TFE3 was also confirmed by live-imaging using GFP-TFE3 (Movie 1). To determine whether dephosphorylation of TFE3 occurred in the cytoplasm or in the nucleus, we examined the time course of dephosphorylation and nuclear translocation by immunoblotting (Fig. 2E).

Dephosphorylated TFE3 appeared in the cytoplasmic fraction 30 min after monensin treatment (lane 5), and most TFE3 was dephosphorylated and still localized in the cytoplasmic fraction at 60 min (lane 8), whereas most TFE3 was found in the nuclear fraction at 90 min (lane 12), suggesting that dephosphorylation of TFE3 occurs in the cytoplasm, and that dephosphorylation precedes nuclear translocation. We confirmed this by examining the time course of nuclear translocation with the use of immunocytochemistry (Fig. 2F). Nuclear translocation of TFE3 was not complete until 3 h after monensin treatment.

To identify phosphorylation sites that anchor TFE3 in the cytoplasm, a series of deletion mutants of TFE3 was expressed in HeLa cells. When TFE3-myc was expressed in HeLa cells, most TFE3-myc was phosphorylated and retained in the cytoplasm in the absence of monensin (Fig. 3A, lanes 1 and 2; Fig. 3B, upper panels), whereas a considerable portion of TFE3-myc was dephosphorylated and translocated to the nucleus after treatment with monensin (Fig. 3A, lanes 3 and 4; Fig. 3B, lower panels), like endogenous TFE3 (Fig. 2D). When a deletion mutant (TFE3 [78-575]-myc) whose N-terminal 77 amino acid residues was deleted was expressed in cells, this mutant was still highly phosphorylated and localized in the cytoplasm (Fig. 3C, lanes 1 and 2; Fig. 3D, upper panels), and it was dephosphorylated and translocated into the nucleus after monensin treatment (Fig. 3C, lanes 3 and 4; Fig. 3D, lower panels). On the contrary, when TFE3 [116-575]-myc was expressed, only a constitutively phosphorylated band (TFE3-1) and a non-phosphorylated band (TFE3-0) were observed (Fig. 3E), and most TFE3 [116-575]-myc was localized in the nucleus even in the absence of monensin (Fig. 3F), suggesting that the phosphorylation sites that are required for cytoplasmic anchoring of TFE3 are present in the [78-115] region.

A series of point mutants of each serine, threonine and tyrosine residue in the [78-115] region were constructed and expressed in HeLa cells. A point mutant in which serine 108 was replaced by alanine (TFE3-S108A) was found to be hardly multi-phosphorylated (Fig. 3G) and was constitutively localized in the nucleus even in the absence of monensin (Fig. 3H), whereas all other point mutants behaved similarly to wild-type TFE3 (Fig. S3). A residually phosphorylated band observed in TFE3-S108A (corresponding to TFE3(L)-2: arrow head in Fig. 3G) may reflect phosphorylation of a minor site. From these observations, we concluded that serine 108 is important for phosphorylation that anchors TFE3 in the cytoplasm. Interestingly, the Golgi apparatus was expanded in cells overexpressing the constitutively active mutant TFE3-S108A (Fig. S4, closed arrow heads) as compared with untransfected control cells (open arrow heads). This suggested that TFE3 is involved in augmentation of the Golgi capacity in accordance with cellular demand.

We also examined whether ER stress affects the phosphorylation status of TFE3. When HeLa cells were treated with canonical ER stress inducers, including thapsigargin and tunicamycin, pATF6(P) was converted to pATF6(N) (Haze et al., 1999), and pXBP1(S) expression was induced (Yoshida et al., 2001) (Fig. SSA and SSB, middle and lower panels, lane 2), indicating that the cells activated the ER stress response. On the contrary, dephosphorylation of
Fig. 3. Phosphorylation status and subcellular localization of deletion and point mutants of TFE3. (A, C, E and G) Phosphorylation status and subcellular localization of TFE3 mutants. Cytoplasmic (Cy) and nuclear (Nu) fractions prepared from HeLa cells expressing the indicated TFE3 mutants were subjected to immunoblotting with an anti-myc antibody. (B, D, F and H) Subcellular localization of TFE3 mutants. Cells expressing the indicated TFE3 mutants were treated with 5 μM monensin for 3 h, and subjected to immunocytochemistry using an anti-myc antibody. Data for other point mutants are shown in Fig. S3. Bars, 10 μm.
TFE3 was not increased by these treatments (upper panel, lane 2). Moreover, we examined subcellular localization of TFE3 during ER stress and found that TFE3 did not translocate into the nucleus upon either thapsigargin or tunicamycin treatment (Fig. S5C, panels a1–c3), while ATF6α and XBP1 were accumulated in the nucleus (panels d1–i3). From these results, we concluded that ER stress did not activate TFE3.

Disruption of Golgi function activates TFE3

To confirm that the signal activating TFE3 originates from the Golgi apparatus, we investigated whether expression of a dominant negative form of GCP60 (GCP60-DN) induced nuclear translocation of TFE3. GCP60 is a Golgi-associated protein whose expression is increased upon Golgi stress by means of the GASE (Oku et al., 2011), and ectopic expression of GCP60 or GCP60-DN causes disassembly of the Golgi structure and blocked Golgi function (Oku et al., 2011; Sohda et al., 2001). When an expression plasmid of GCP60-DN was transiently transfected into HeLa cells, the Golgi apparatus detected by anti-Giantin antiserum became disrupted in appearance (Fig. 4A, closed arrow heads), whereas the Golgi apparatus in control cells was localized in the perinuclear region (open arrow heads). In these Golgi-disrupted cells expressing GCP60-DN, TFE3 was concentrated in the nucleus (Fig. 4B, closed arrow heads), but TFE3 was retained in the cytoplasm in cells that did not express GCP60-DN (open arrow heads), indicating that expression of GCP60-DN caused the activation of TFE3. We speculated that overexpression of GCP60-DN caused disruption of the Golgi structure, which attenuated Golgi function including glycosylation and vesicular transport, resulting in the activation of TFE3. Expression of GCP60-DN was confirmed by immunoblotting (Fig. 4C, lane 2).

Inhibition of glycosylation of proteoglycans activates TFE3

Because GCP60-DN seemed to attenuate overall Golgi function by disrupting Golgi structure, it was unclear what kinds of molecular changes in the Golgi activate the Golgi
stress response. To investigate whether insufficiency of oligosaccharide processing in the Golgi apparatus is responsible for activation of TFE3, the effect of specific inhibition of protein glycosylation was examined. 4-Methylumbelliferyl-beta-D-xylolside (xylolside) is an inhibitor of O-glycosylation of core proteins of proteoglycans (Hamati et al., 1989). It has been reported that xylolside treatment caused accumulation of proteoglycans in the Golgi apparatus and vesicularization of the Golgi (Kanwar et al., 1986), and it also stimulated the synthesis of proteoglycan core proteins (Kanwar et al., 1984), suggesting that xylolside may cause disruption of Golgi function and induce the Golgi stress response. When cells were treated with xylolside, the dephosphorylation rate of TFE3(L) and TFE3(S) was increased by 2.7- and 3.3-fold, respectively (Fig. 5A, lane 2), whereas nuclear translocation of TFE3(L) and (S) was upregulated by 6.4- and 19.8-fold, respectively (Fig. 5B, panels d-f and Fig. 5C). Moreover, when cells transfected with a GASE-LUC reporter plasmid were treated with xylolside, transcription from GASE increased by 9.7-fold (Fig. 5D, lane 2). Knockdown of TFE3 expression by siRNAs resulted in reduced transcriptional induction in response to xylolside (from 4.1-fold to 1.4-fold by siTFE3-A, and from 4.4-fold to 1.2-fold by siTFE3-B) (Fig. 5E, lanes 4 and 8). These findings suggested that inhibition of proteoglycan glycosylation leads to activation of the TFE3-GASE pathway of the mammalian Golgi stress response. To confirm that insufficiency of proteoglycan glycosylation leads to activation of the TFE3-GASE pathway, we analyzed sog9, a mutant cell line that has a defect in glycosylation of proteoglycan (Banfield et al., 1995). Production of HS and chondroitin sulfate (CS) was reduced to 3.9% and 36.2% respectively in sog9 cells due to the dysfunction of EXT1 and C4ST-1 (Fig. 6A) (Banfield et al., 1995; Uyama et al., 2006), which encode a glycosyltransferase essential for the synthesis of HS chains and chondroitin 4-O-sulfotransferase-1 for transferring a sulfate to the 4-O-position of a GalNAc residue in CS chains, respectively (Nadanaka and Kitagawa, 2008; Uyama et al., 2006). In sog9 cells, the phosphorylation rate of TFE3(L) and TFE3(S) was decreased to 48% and 50%, respectively (Fig. 6B and 6C), and expression of GCP60 mRNA, a target gene of TFE3, was increased by 1.6-fold (Fig. 6D, lane 2), as compared with parental L cells. Sog9-C4ST-1-1 and sog9-C4ST-1-5 cells are stable lines of sog9 cells that were stably transfected with an expression plasmid for C4ST-1 and express C4ST-1 weakly and strongly, respectively (Uyama et al., 2006). In these stable cell lines, the dephosphorylation rate of TFE3(S) was decreased by 56.9% in C4ST-1-1 and 71.2% in C4ST-1-5 (Fig. 6E, lanes 2 and 3 and Fig. 6F, lanes 5 and 6), and induction of GCP60 mRNA expression was compromised compared with in sog9 cells (decreased to 34.5% and 73.1% for Sog9-C4ST-1-1 and Sog9-C4ST-1-5, respectively) (Fig. 6G, lanes 2 and 3). These results supported the notion that a deficiency in proteoglycan glycosylation in the Golgi apparatus causes Golgi stress and activates TFE3. Because rescue of HS synthesis decreased the expression of GCP60 mRNA (Fig. 6G), it is possible that the contribution of the inhibition of HS synthesis is smaller than that of CS synthesis. However, because we did not evaluate the effect of rescue of HS synthesis, it is still possible that HS synthesis is as important as CS synthesis. It is also possible that lack of sulfation is more important for induction of Golgi stress. The precise molecular nature of Golgi stress is an interesting subject, which will be analyzed in the future.

**Inhibition of sialic acid modification of proteins and lipids activates TFE3**

Sialic acid modification of N-glycans, O-glycans and lipids occurs in the Golgi apparatus, using CMP-sialic acid (Varki et al., 2008). SLC35A1 is a nucleotide-sugar transporter that transports CMP-sialic acid from the cytosol to the Golgi apparatus (Nishihara, 2014). It has been reported that suppression of SLC35A1 expression by siRNA resulted in attenuation of sialic acid modification of proteins (Xu et al., 2010). To examine whether insufficiency of sialic acid modification activates TFE3, we first checked the suppression efficiency of SLC35A1 siRNAs (siSLC35A1-A and -B) by qRT-PCR (Fig. 7A), and found that siSLC35A1-A and -B efficiently reduced the expression of SLC35A1 mRNA to 23.2% and 5.9%, respectively (lanes 2 and 3). In SLC35A1 knockdown cells, the dephosphorylation rate of TFE3(L) was increased by 3.0- and 2.1-fold by siSLC35A1-A and -B, respectively (Fig. 7B, upper panel, lanes 2 and 3, and Fig. 7C) as compared with control cells (lane 1), whereas that of TFE3(S) was upregulated by 2.5- and 1.4-fold, respectively. In addition, TFE3 was concentrated in the nucleus in SLC35A1 knockdown cells (Fig. 7D, panels d–i). Moreover, the transcription of target genes of the Golgi stress response, including GCP60, SIAT4A and FUT1, was induced after SLC35A1 knockdown by 2.8- and 8.7-fold (GCP60), 4.0- and 5.6-fold (SIAT4A), and 2.6- and 4.8-fold (FUT1) by siSLC35A1-A and -B, respectively (Fig. 7E, lanes 2, 3, 5, 6, 8 and 9). These results suggested that insufficiency of sialic acid modification in the Golgi apparatus can activate the Golgi stress response.

**mTOR is not essential for activation of TFE3 by Golgi stress**

It is possible that core proteins of proteoglycans lacking oligosaccharides or glycoproteins lacking sialic acid modification accumulate in the Golgi, cause Golgi dysfunction and induce autophagy of the Golgi apparatus, and then the signal inducing autophagy indirectly activates TFE3. To exclude this possibility, we investigated whether a kinase, mTOR, whose activity is known to be suppressed during autophagy and as such is a good marker for initiation of
Fig. 5. Disruption of glycosylation of proteoglycans in the Golgi apparatus by xyloside activates the TFE3-GASE pathway. (A) Phosphorylation status of TFE3 upon xyloside treatment. HeLa cells treated with 7.5 mM xyloside for 16 h were subjected to immunoblotting with anti-TFE3-B antiserum. The ratio between less phosphorylated TFE3 (TFE3-0 and 1) and highly phosphorylated TFE3 (TFE3-2 and 3) is shown in the middle and lower panels. (B and C) Subcellular localization of TFE3 after xyloside treatment. HeLa cells treated with 7.5 mM xyloside for 16 h were subjected to immunocytochemistry with anti-TFE3-B antiserum and DAPI (B), or fractionated into nuclear and cytoplasmic fractions for immunoblotting (C). Bars, 10 μm. (D) Transcriptional induction from the GASE after xyloside treatment. HeLa cells transfected with a GASE-luciferase reporter were treated with 7.5 mM xyloside for 18 h and subjected to luciferase assays. The activity in lane 1 is set as 1. (E) Effect of siTFE3 on xyloside-induced transcription from GASE. HeLa cells transfected with a GASE-luciferase reporter and siTFE3 were treated with 4.5 mM xyloside for 22 h and subjected to luciferase assays. The activity in lanes 1 and 5 is set as 1.
autophagy (Laplante and Sabatini, 2012), is inactivated upon inhibition of oligosaccharide processing. S6 kinase (S6K) is a well known substrate of mTOR and, when autophagy is induced, S6K becomes dephosphorylated, leading to attenuation of translation (Magnuson et al., 2012). S6K was completely dephosphorylated when autophagy was induced by amino acid starvation (Fig. S6A, lane 3). In contrast, even if genes encoding enzymes required for proteoglycan glycosylation were disrupted (lane 2), S6K was hardly dephosphorylated. In addition, when Golgi stress was induced by suppression of SLC35A1 expression, S6K was hardly dephosphorylated (Fig. 7B, lanes 2 and 3). These observations suggested that activation of TFE3 by disruption of Golgi function is independent of induction of autophagy.

We also investigated the effect of mTOR knockdown on TFE3 activity. We prepared three siRNAs for mTOR (si-mTORs), which effectively suppressed expression of mTOR as well as phosphorylation of S6K (Fig. S6B, upper and lower panels, lanes 2–4). When HeLa cells were transfected with these si-mTORs, TFE3 was not dephosphorylated (middle panels) and did not translocate to the nucleus (Fig. S6C, panels d–l). Moreover, when the effect of si-mTORs was evaluated with the use of a GASE-LUC reporter, we found that transcription from GASE was not induced by mTOR knockdown (Fig. S6D, lanes 4–6), suggesting that mTOR inactivation, which induces autophagy, is not sufficient for TFE3 activation. From these results, we concluded that Golgi dysfunction, such as insufficiency of oligosaccharide processing, activates the TFE3-GASE pathway without the involvement of mTOR or autophagic signals. This result is in agreement with an observation by Smith and colleagues that mTOR activates TFE3 through the inactivation of Fln (Betschinger et al., 2013).

Fig. 6. Disruption of proteoglycan glycosylation by genetic mutation activates the TFE3-GASE pathway. (A) Proteoglycan expression in sog9 cells. Heparan sulfate (HS) and chondroitin sulfate (CS) extracted from L and sog9 cells were measured by HPLC. (B) Phosphorylation status of TFE3 in sog9 cells. Whole cell lysates prepared from L and sog9 cells were subjected to immunoblotting with an anti-TFE3-B antiserum. Image of lane 1 was obtained by slightly enhancing exposure as compared with lane 2. (C) Data in (B) were quantified. The value in lanes 1 and 3 is set as 1. (D) Effect of sog9 mutation on expression of the GCP60 gene. Expression of GCP60 mRNA in L and sog9 cells was measured by qRT-PCR. The expression in lane 1 is set as 1. (E–G) Rescue experiments for the sog9 mutation. Expression levels of TFE3 (E) and GCP60 mRNA (G) in sog9 cells transfected with a C4ST-I expression vector were examined as in (B) and (D). Quantitated data from (E) was shown in (F).
Fig. 7. Disruption of the CMP-sialic acid transporter activates the TFE3-GASE pathway. (A) Knockdown efficiency of SLC35A1 by siRNAs. RNA was prepared from HeLa cells transfected with siRNAs for SLC35A1 (siSLC35A1-A and siSLC35A1-B) and subjected to qRT-PCR with primers for SLC35A1. The expression in lane 1 is set as 1. (B) Phosphorylation status of TFE3 in SLC35A1 knockdown cells. HeLa cells were transfected with siSLC35A1-A or -B and subjected to immunoblotting with anti-TFE3-B, anti-phosphorylated S6K, anti-S6K, and anti-β-tubulin antisera. Data were from the same blot. (C) Quantitated data from (B). The ratio between dephosphorylated (TFE3-0 and TFE3-1) and phosphorylated TFE3 (TFE3-2 and TFE3-3) is shown. Data were obtained from triplicate experiments. (D) Subcellular localization of TFE3 in SLC35A1 knockdown cells. HeLa cells were transfected with siSLC35A1-A or -B and subjected to immunocytochemistry with anti-TFE3-B antiserum and DAPI. Bars, 10 μm. (E) Effect of SLC35A1 knockdown on the expression of target genes of the Golgi stress response. RNA was prepared from HeLa cells transfected with siSLC35A1-A or -B, and subjected to qRT-PCR with primers for the indicated genes. The expression in lanes 1, 4 and 7 is set as 1.
Discussion

In the present study, we revealed a core mechanism regulating the mammalian Golgi stress response (Fig. 8). In normal growth conditions, a key transcription factor, TFE3, is phosphorylated and anchored in the cytoplasm. When the capacity of the Golgi becomes insufficient, TFE3 is dephosphorylated, translocates into the nucleus, binds to the GASE enhancer element and activates the transcription of Golgi-related genes, resulting in upregulation of Golgi function. Our findings not only provide a basis for the analysis of the Golgi stress response, but also contribute to establish the novel concept of organelle autoregulation.

Recently, several laboratories published papers related to the Golgi stress response. Tohyama and colleagues reported that inhibition of O-GalNAc glycosylation in the Golgi apparatus by treatment with benzyl-GalNAc induces the transcription of HSP47, a molecular chaperone that assists folding of collagens in the ER (Miyata et al., 2013). In addition, Sabatini and colleagues reported that treatment with brefeldin A, which causes absorption of the Golgi apparatus to the ER and induces ER stress (Citterio et al., 2008; Islam et al., 2006; Moon et al., 2012), activates an ER-bound transcription factor CREB3/Luman and induces transcription of a small GTPase ARF4, resulting in apoptosis (Reiling et al., 2013). These signaling pathways may represent part of the Golgi stress response pathways and are clearly distinct from the TFE3-GASE pathway, which regulates the expression of glycosylation enzymes, vesicular transport components and Golgi structural proteins. These pathways collectively maintain homeostasis of the Golgi apparatus by regulating the distinct aspects of Golgi function.

MITF subfamily and organelle autoregulation

TFE3 belongs to the MITF subfamily of mammalian bHLH-ZIP transcription factors, which consists of MITF, TFEB, TFE3 and TFEC (Atchley and Fitch, 1997), and these transcription factors seem to be involved in organelle autoregulation. MITF is a well-characterized transcription factor essential for melanosome biogenesis. The activity of MITF is upregulated by its transcriptional induction as well as phosphorylation of the MITF protein by p38, ERK2 and GSK3β (Steingrimsson et al., 2004). TFEB, another member of the MITF subfamily, is essential for the biogenesis of lysosomes. TFEB is phosphorylated by mTORC1 and retained in the cytoplasm in normal growth conditions, whereas TFEB was dephosphorylated upon amino acid starvation, translocated into the nucleus, and activated the transcription of genes involved in lysosomal biogenesis as well as autophagy (Pena-Llopis et al., 2011; Sardiello et al., 2009; Settembre et al., 2012).

Interestingly, TFE3 has a distant cousin in budding yeast, RTG3, which is also involved in organelle autoregulation, that is, mitochondrial retrograde signaling (Atchley and Fitch, 1997). RTG3, a bHLH-ZIP transcription factor, is phosphorylated and anchored in the cytoplasm in normally growing cells, whereas RTG3 is dephosphorylated when the function of the mitochondria is compromised, and translocated into the nucleus with another bHLH-ZIP transcription factor RTG1, which lacks the transcriptional activation domain (Liu and Butow, 2006). RTG3-RTG1 heterodimers activate transcription involved in the TCA cycle to enhance respiratory function in the mitochondria. Since the regulatory mechanisms of TFEB and RTG3 are similar to that of TFE3, it is possible that these evolved from the same ancestral mechanism in order to regulate autoregulation of each organelle.

The ER stress response and the Golgi stress response are autoregulatory mechanisms controlling the capacity of the ER and the Golgi apparatus in accordance with cellular demands to maintain their homeostasis when the capacity of these organelles is overwhelmed by increased synthesis of secretory proteins. Such an autoregulatory mechanism may exist in other organelles. The lysosome stress response regulated by TFEB, and peroxisomal proliferation controlled by a transcription factor PPARα (Phelps et al., 2006) can be considered as organelle autoregulation. The mitochondrial retrograde response regulated by RTG3 in yeast,
the mitochondria stress response controlled by a transcription factor CHOP in mammals (Ryan and Hoogenraad, 2007) and the mitochondrial unfolded protein response regulated by transcription factors DVE-1 and ATF6-1 in Caenorhabditis elegans (Haynes et al., 2007, 2010; Nargund et al., 2012) may be a part of an autoregulatory mechanism of mitochondria. These autoregulatory mechanisms of organelles are indispensable for eukaryotic cells to maintain intracellular homeostasis.

**What kinds of molecular changes activate the Golgi stress response?**

It was initially unclear what kinds of molecular changes occur in the Golgi apparatus during Golgi stress. In the case of ER stress, unfolded proteins accumulate in the ER when ER function becomes insufficient, and sensors of ER stress become activated in response, including ATF6, PERK and IRE1 (Ron and Walter, 2007). Thapsigargin and the calcium ionophore A23187 are usually used to artificially induce ER stress in experimental systems, which deplete Ca\(^{2+}\) ions from the lumen of the ER and reduce the activity of ER enzymes, including ER chaperones, because Ca\(^{2+}\) ions are required for the activity of ER chaperones (Suzuki et al., 1991). Monensin, a Golgi stress inducer used in the present study, is an ionophore that depletes protons from acidic compartments including the Golgi apparatus, lysosomes and endosomes. Because an acidic environment is indispensable for the activity of Golgi enzymes, monensin effectively reduces the capacity of Golgi functions such as glycosylation and vesicular transport (Daniels and Edwardson, 1988; Dinter and Berger, 1998; Lipsky and Pagano, 1985; Tartakoff and Vassalli, 1977), leading to the induction of Golgi stress. However, monensin is not specific to the Golgi apparatus.

We observed that expression of GCP60-DN, which causes fragmentation of the Golgi apparatus and blockade of vesicular transport (Sohda et al., 2001) (Fig. 4A), induced nuclear translocation of TFE3 (Fig. 4B), suggesting that the activation signal of TFE3 comes from the disrupted Golgi apparatus. Although GCP60 has not been characterized extensively, it is crucial for Golgi structure and function. GCP60 interacts with giantin via its C-terminus GOLD domain (Sohda et al., 2001), and their interaction is crucial for maintenance of the Golgi structure and function. GCP60 also interacts with phosphatidylinositol 4-kinase III beta to regulate vesicular transport from the trans-Golgi compartment by synthesizing phosphatidylinositol 4-phosphate (Sasaki et al., 2012). Moreover, GCP60 is involved in neurodegenerative diseases, including Huntington’s disease (Sbodio et al., 2013).

We also revealed that perturbation of oligosaccharide processing in the Golgi apparatus activates the Golgi stress response (Fig. 5, Fig. 6 and Fig. 7), suggesting that the glycosylation status of secreted proteins in the Golgi apparatus is important for activation of the TFE3-GASE pathway of the mammalian Golgi stress response. Core proteins lacking proper oligosaccharides may accumulate in the Golgi apparatus and activate the Golgi stress response (Kanwar et al., 1986), although it is also possible that lectins recognize insufficient glycosylation and activate the Golgi stress response. Identification of the sensor molecules that detect Golgi stress could reveal the molecular nature of Golgi stress as well as the molecular mechanism of stress sensing.

Finally, we revealed that autophagic signals are not directly involved in the activation of TFE3 (Fig. S6), which is consistent with previous findings that monensin treatment did not induce the transcription of genes involved in autophagy and lysosomes (Oku et al., 2011). Of course, it could be possible that there is crosstalk between the Golgi stress response and autophagy. In the case of the ER, unfolded proteins accumulating in the ER are disposed of by ER-specific autophagy (ER-phagy), which is induced by the ER stress response (Bernales et al., 2007). Thus, it is possible that severely dysfunctional Golgi apparatus suffering from prolonged Golgi stress is disposed of by Golgi-specific autophagy (Golgi-phagy), which might be induced by the Golgi stress response. Crosstalk between the Golgi stress response and autophagy is an interesting topic for future research.

**Physiological function of the TFE3-GASE pathway**

It has been reported that TFE3 is involved in various physiological phenomena, including B-cell activation (Merrell et al., 1997), osteoclast or mast cell differentiation (Steingrimsson et al., 2002; Yagil et al., 2012), expression of the CD40 ligand (Huan et al., 2006), insulin signaling (Nakagawa et al., 2006), tumors (Hong et al., 2010) and pluripotency (Betschinger et al., 2013). It might be possible that TFE3 is required for homeostasis of the Golgi apparatus in these processes, because synthesis of secretory proteins is an important part of them.

Therefore, what is the physiological function of the Golgi stress response in organisms? We speculated that eukaryotic cells augment Golgi capacity to support the differentiation of professional secretory cells such as goblet cells and pancreatic acinar cells, and the Golgi stress response is important for this process like the ER stress response. Moreover, because the Golgi apparatus is closely linked to various diseases, including neurodegenerative diseases (Dal Canto, 1996; Gonatas et al., 1998; Hu et al., 2007; Sun et al., 2008), it is highly possible that the Golgi stress response is involved in Golgi-related diseases. Our current findings on the TFE3-GASE pathway would provide a basis for the analysis of physiological function of the Golgi stress response.

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TFE3 Regulates Mammalian Golgi Stress Response

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