UNIVERSITY OF HYOGO

ANALYSIS OF THE MUCIN PATHWAY OF THE MAMMALIAN GOLGI STRESS RESPONSE

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ABSTRACT

The Golgi plays a central role for post-translational modifications of properly folded proteins from the ER (e.g. glycosylation and sulfation) as well as vesicular transport of correctly modified proteins to their final destination. When the synthesis of secretory or membrane proteins is increased and overwhelms the functional capacity of the Golgi apparatus (Golgi stress), eukaryotic cells activate a homeostatic mechanism called the Golgi stress response in order to upregulate the capacity of the Golgi. Recent studies have revealed five Golgi stress response pathways such as the TFE3, CREB3, HSP47, MAPK-ETS and proteoglycan pathways, which regulates the general function of the Golgi, apoptosis, cell survival, spliceosome and cell death, and glycosylation of proteoglycans, respectively. Here, we have uncovered a novel response pathway, named the mucin pathway, that enhances the expression of glycosylation enzymes for mucins (such as GALNT5, GALNT8 and GALNT18) to compensate for the insufficiency in mucin-type glycosylation in the Golgi (mucin-type Golgi stress). Unexpectedly, we found that TFE3, a key transcription factor regulating the TFE3 pathway, was also induced and activated upon mucin-type Golgi stress, suggesting that the mucin pathway transmits a crosstalk signal to the TFE3 pathway. Promoter analysis of the human TFE3 gene revealed a novel enhancer element that regulates transcriptional induction of the TFE3 gene in response to mucin-type Golgi stress, of which consensus sequence is ACTTCC(N9)TCCCCA. We named this sequence the mucin-type Golgi stress response element (MGSE). These observations suggested that crosstalk signal from the mucin pathway to the TFE3 pathway plays an important role in the regulation of the mammalian Golgi stress response.

CONTENTS

LIST OF ABBREVIATONS

Abbreviations

AARE	amino acid starvation response element
ARF4	ADP-ribosylation factor 4
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
BenzylGalNAc	benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside
BFA	Brefeldin A
BG	benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside
cas9	CRISPR associated protein 9
cDNA	complementary DNA
CGN	cis-Golgi network
CREB3	cyclic AMP-responsive element-binding protein 3
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
ERAD	ER-associated degradation
ERSE	ER stress response element
ETS	E26 transformation-specific
GAL4	galactose nonutilizer 4
GalNAc	N-acetylgalactosamine
GALNT1	polypeptide N-acetylgalactosaminyltransferase 1
GALNT5	polypeptide N-acetylgalactosaminyltransferase 5
GALNT8	polypeptide N-acetylgalactosaminyltransferase 8
GALNT18	polypeptide N-acetylgalactosaminyltransferase 18
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GASE	Golgi apparatus stress response element
GCP60	Golgi complex-associated protein of 60 kDa
GM130	cis-Golgi matrix protein of 130k Da
GOMED	Golgi membrane-associated degradation pathway

GPI	Glycosylphosphatidylinositol
HSP47	heat shock protein 47 kDa
IgA	immunoglobulin A
IRE1	inositol-requiring protein 1
MAPK	Mitogen-activated protein kinase
MGSE	mucin-type Golgi stress response element
MUC1	mucin 1
MUC20	mucin 20
NF-Y	nuclear transcription factor Y
PERK	PKR-like ER kinase
PGSE	proteoglycan stress response element
qRT-PCR	quantitative reverse transcription PCR
RNA-Seq	RNA sequencing
S1P	membrane-bound transcription factor site-1 protease
S2P	membrane-bound transcription factor site-2 protease
TFE3	transcription factor E3
TGN	trans-Golgi network
Tn	GalNAcα1-O-Ser/Thr (glycosidic bond)
UPRE	unfolded protein response element
UDP-GalNAc	Uridine 5′-diphospho-N-acetylgalactosamine
XBP1	X-box binding protein 1
Y1H	Yeast one hybrid screening

CHAPTER ONE
INTRODUCTION

1.1 Organelle Autoregulation

Eukaryotic cells have various organelles such as the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, lysosomes and peroxisomes. The amount of each organelle appears to be separately and strictly regulated in accordance with cellular needs by the mechanism of organelle autoregulation (Sasaki and Yoshida, 2015). Upon cell division, the quantity of each organelle is multiplied accordingly to keep its quantity constant. Intriguingly, when cells require more function of a particular organelle due to insufficiency of its capacity (organelle stress), cells activate the homeostatic mechanism in order to specifically augment the amount of the organelle (Fig. 1). For instance, during the differentiation of plasma cells, the ER is expanded to support folding of huge amount of secretory and membrane proteins (Wiest *et al.*, 1990). Similarly, during differentiation of secretory mucous cells of Brunner's gland the Golgi apparatus is extensively expanded (Fig. 2) (Berger and Roth, 1997). Thus, these suggest that organelle autoregulation is an important homeostatic mechanism to adjust the capacity of each organelle to the cellular demands.

Organelle autoregulation is indispensable for eukaryotic cells in order to regulate cellular functions, and it is one of the fundamental issues in cell biology. In principle, organelle autoregulation consists of four factors: (1) sensors that detect insufficiency of organelle function, (2) transcription factors that upregulate the expression of genes related to organelle function, (3) enhancer elements to which the transcription factors bind, and (4) target genes whose expression is augmented by organelle autoregulation, resulting in upregulation of organelle capacity (Fig. 1) (Sasaki and Yoshida, 2019). Focusing on the Golgi apparatus, this section will emphasize the essentials of the molecular mechanism of the Golgi stress response and its response pathways. Nevertheless, it is extremely important to describe about the ER stress response since it has been well-characterized in the previous studies and other organelles seem to have fundamentals similar to the ER stress response.

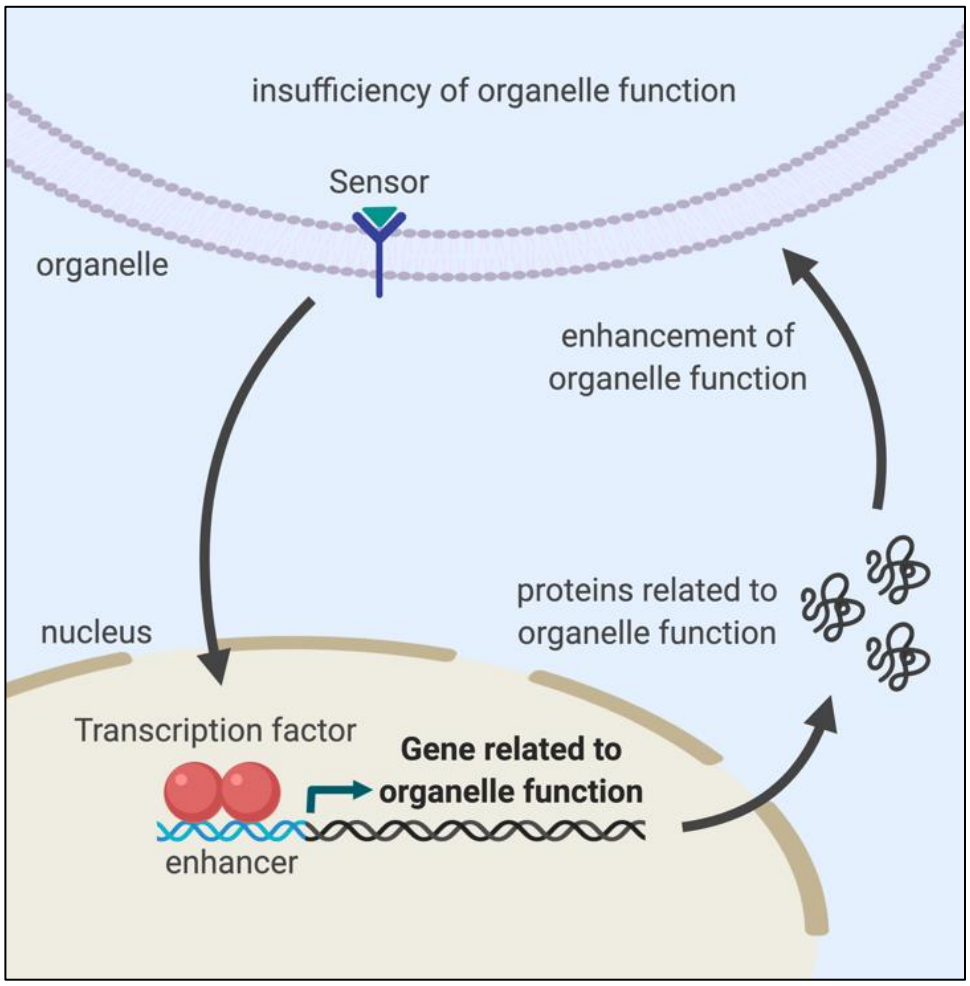


Fig. 1 The fundamentals of organelle autoregulation

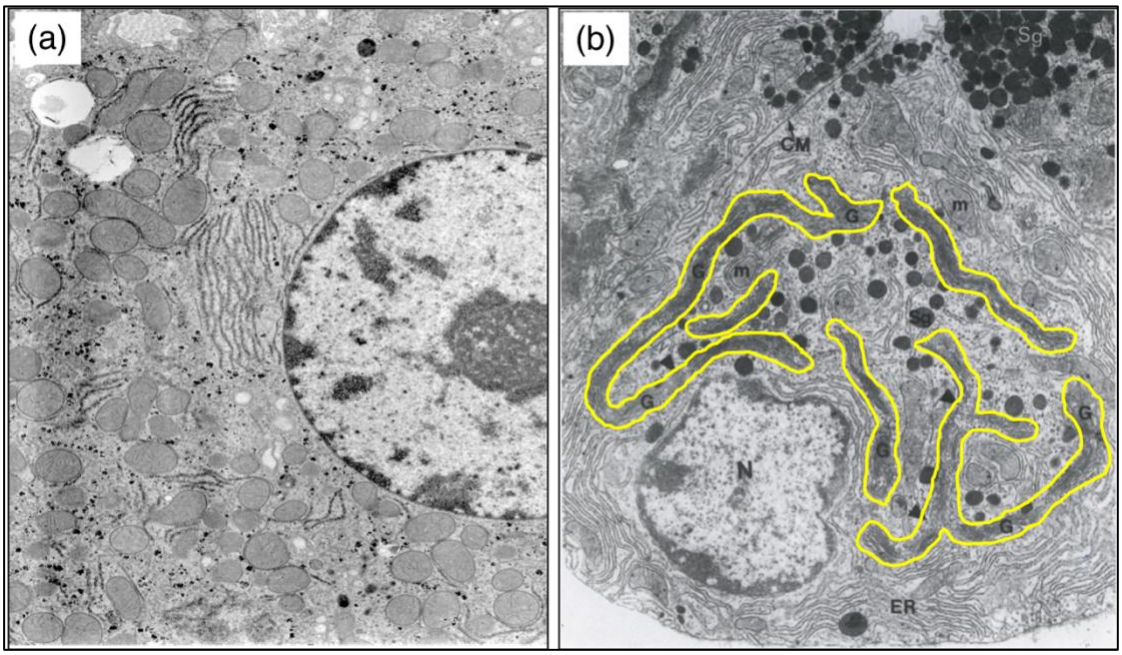


Fig. 2. The amount of the Golgi in hepatocytes (a) is almost negligible compared to that in Brunner's gland mucous secreting cells (b) (Mol. bio. of the cell, 5th ed., Alberts *et al.*, 2002)

1.2 The Endoplasmic Reticulum (ER) Stress Response

There are three distinct, well-conserved pathways in the mammalian ER stress response: the ATF6, IRE1 and PERK pathways. The ATF6 pathway regulates expression of ER chaperone genes (Okada *et al.*, 2002; Yamamoto *et al.*, 2007), whereas the IRE1 pathway augments expression of factors for ER-associated degradation (Calfon *et al.*, 2002; Cox *et al.*, 1993; Mori *et al.*, 1993; Shen *et al.*, 2001; Hiderou Yoshida *et al.*, 2001; Yoshida *et al.*, 2003). The PERK pathway controls ER stress-induced apoptosis (Harding *et al.*, 1999a, 2000; Yan *et al.*, 2002). As mentioned in the concept of 'organelle autoregulation', each pathway regulating the ER stress response has (1) a sensor that detects insufficiency of ER function (accumulation of unfolded proteins in the ER, which is called ER stress), (2) a transcription factor that binds to an enhancer element, (3) a specific enhancer element that lies in the promoter regions of the target genes, and (4) target genes of which expression is upregulated to relieve ER stress. In the case of the ATF6 pathway, the sensor, the transcription factor, the enhancer element and the target genes are pATF6(P), pATF6(N), ERSE and ER chaperone genes, respectively (Haze *et al.*, 1999; Hiderou Yoshida *et al.*, 2001; Yoshida *et al.*, 1998, 2000), and those of the IRE1 pathway are IRE1, pXBP1(S), UPRE and ERAD genes, respectively (Calfon *et al.*, 2002; Shen *et al.*, 2001; Yamamoto *et al.*, 2008; Hiderou Yoshida *et al.*, 2001; Yoshida *et al.*, 2003). As for the PERK pathway, PERK is the sensor, ATF4 is the transcription factor, AARE is the enhancer element and apoptosis-associated factors are the target genes (Harding *et al.*, 1999b, 2000). The essentials of the ER stress response are summarized in Fig. 3 (Taniguchi and Yoshida, 2017).

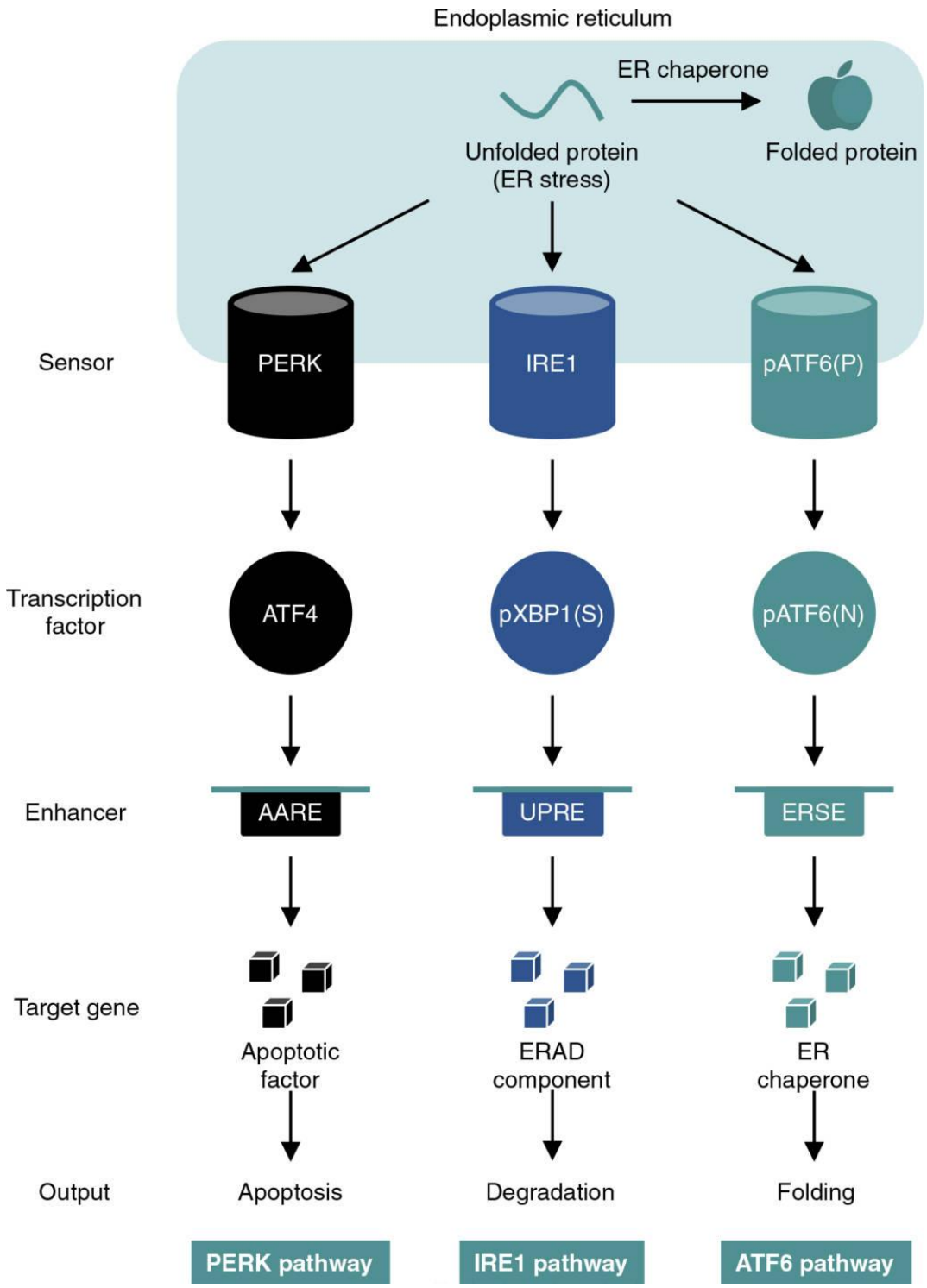


Fig. 3. There are three mammalian ER stress response pathways: the IRE1, PERK and ATF6 pathways as described in the text. (Taniguchi and Yoshida, 2017)

1.3 The Golgi Stress Response and Organelle Zone

The Golgi apparatus is an organelle where posttranslational modification of secretory and membrane proteins synthesized in the ER takes place before they are selectively transported to their final destination. When large amount of secretory and membrane proteins are transported from the ER to the Golgi, they can overwhelm the functional capacity of the Golgi, leading to Golgi stress and activation of the Golgi stress response. Compared to the ER stress response, the Golgi stress response has been less studied but the principles are still the same. In this section, we will briefly describe the recent works of the Golgi stress response and organelle zones that has been proposed residing within all organelles including the Golgi.

1.3.1 The Golgi Stress Response Pathways

Thus far, at least five response pathways of the Golgi stress response have been revealed in mammalian system, namely the TFE3, CREB3, HSP47, MAPK-ETS and proteoglycan pathways as shown in Fig. 4. TFE3 is a key transcription factor regulating the TFE3 pathway. In normal growth conditions, TFE3 is phosphorylated and retained in the cytoplasm. Upon Golgi stress, TFE3 is dephosphorylated, translocates into the nucleus and activates the transcription of target genes by binding to the Golgi apparatus stress response element (GASE) (Taniguchi *et al.*, 2015, 2016). The target genes of the TFE3 pathway include N-glycosylation enzymes, Golgi structural proteins, and vesicular transport components (Oku *et al.*, 2011), suggesting that the TFE3 pathway augments general function of the Golgi. The sensor molecule for the TFE3 pathway has been yet to be discovered.

The CREB3 pathway was identified by Reiling and colleagues (Reiling *et al.*, 2013), of which key transcription factor is CREB3. CREB3 is a transmembrane protein located in the ER membrane and functions as a sensor for Golgi stress, whereas upon Golgi stress CREB3 is translocated from the ER to the Golgi and is cleaved by S1P and S2P proteases. The cytosolic portion of cleaved CREB3 translocates to the nucleus and activates transcription of a small GTPase ARF4, resulting in Golgi stress-induced apoptosis. In addition to the CREB3 pathway, Reiling and colleagues also proposed that Golgi stress inducers (BFA, Golgicide A and monensin) could also evoke apoptosis via the mitogen activated protein kinase (MAPK)-ETS pathway (Baumann *et al.*, 2018). They identified three transcription factors (ETS1, ELK1 and GAPBA/B) via

transcription binding motif analysis. Upon Golgi stress, these transcription factors of ETS family are orchestrated by MAPK to induce an apoptotic factor of MCL1, leading to programmed cell death. However, the details of the MAPK-ETS pathway as well as its relationship with the CREB3 pathway still remains obscured.

In contrast to the CREB3 pathway, the HSP47 pathway increases the expression of HSP47, an ER chaperone specialized for folding and maturation of collagens, and suppresses Golgi stress-induced apoptosis (Miyata *et al.*, 2013). However, the sensor, transcription factor, and the enhancer element of the HSP47 pathway remain to be identified. Recently, the proteoglycans (PGs) pathway has been revealed to increase the expression of PG-glycosylation enzymes upon Golgi stress (proteoglycan-type Golgi stress) (Sasaki *et al.*, 2019). PGSE-A and PGSE-B are enhancer elements regulating the proteoglycan pathway, although the transcription factors and sensors has been unidentified. The proteoglycan pathway seems to be a cell type-specific response pathway and to be upregulated in cells producing proteoglycans including chondrocytes and reactive astrocytes (Sakamoto, 2017).

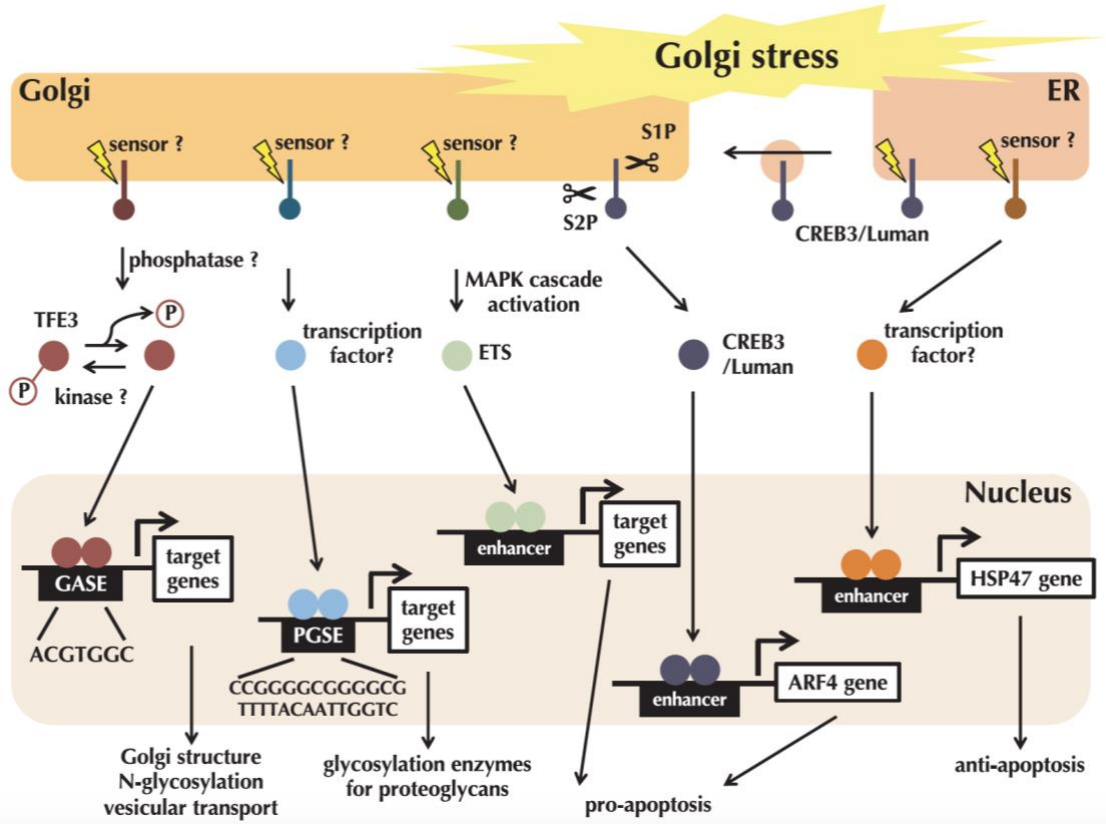


Fig. 4. The recent studies in the mammalian Golgi stress response. At least five pathways have been reported, including TFE3, HSP47, CREB3, MAPK-ETS, and proteoglycan pathways (Sasaki and Yoshida, 2019).

1.3.2 Organelle Zones in the Golgi apparatus

The functional capacity of the Golgi apparatus in its architecture is dynamic and flexible. As proposed by Yoshida and colleagues, various local functional regions exist within an organelle including the Golgi that dictates the organelle function such as the proteoglycan zone, the mucin zone, the GPI zone, the lipid transfer zone, and the degradation zone (Fig. 5) (Sasaki and Yoshida, 2019a). Since these zones are distinct in their function, it is believed that these zones have separate sensors, transcription factors, enhancer elements and target genes. This notion has prompted the analysis of our work to explore the existence of the mucin zone and the mucin pathway of the Golgi stress response that may bring a new perspective in the research of mucus biotherapeutics and mucus-associated diseases.

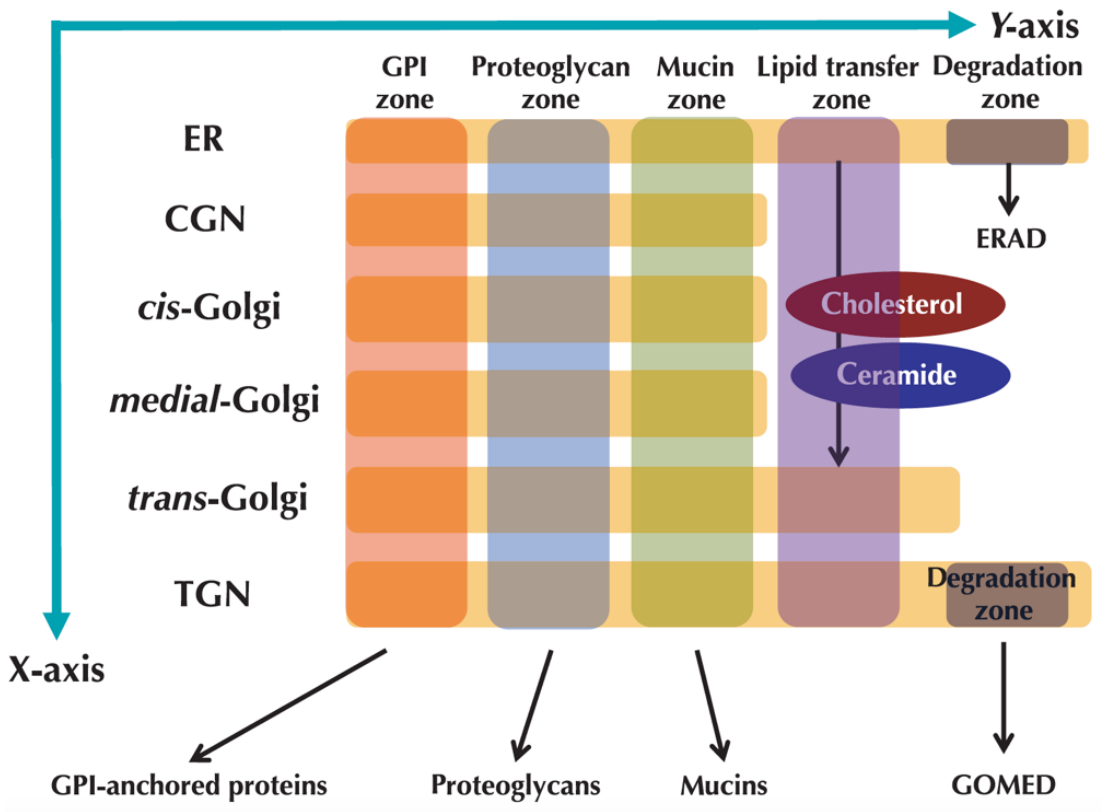


Fig. 5. The organelle zones within the Golgi apparatus as proposed by Sasaki and Yoshida, 2019a

1.4 Analysis of the Mucin Pathway in the Mammalian Golgi Stress Response

Mucus is one of the main products of goblet cells in the columnar epithelium which lines the surface of all important organs that are exposed to the external environment

(Brockhausen and Stanley, 2015). Examples include the gastrointestinal tract, the reproductive tract, the respiratory tract and the oculo-rhino-otolaryngeal tract. Mucus serves multiple functions such as lubrication and defence against foreign noxious stimulus (Allen *et al.*, 1982; Birchenough *et al.*, 2015). While serving as a barrier structure, it also allows the exchange of gas and nutrients, owing to its permeable feature from 95% water composition (Bansil and Turner, 2006).

Mucins, the macromolecular components of mucus are heavily O-glycosylated proteins. They are synthesized through a series of complex glycosylation reactions in the Golgi apparatus which involve various types of modification enzymes (Brockhausen and Stanley, 2015). The glycosylation reaction of mucins initiates with the transfer of GalNAc from UDP-GalNAc to Ser or Thr residue of mucin core proteins, which is catalyzed by polypeptide N-acetyl-a-d-galactosaminyltransferases (GALNTs) that have more than 20 different isoforms (Hang and Bertozzi, 2005; Hanisch, 2001; Steen *et al.*, 1998). Attachment of the initial GalNAc triggers the action of numerous glycosyltransferases in a series of complex glycosylation extending the GalNAc to multiples diversification of different O-glycan structures (Fig. 6).

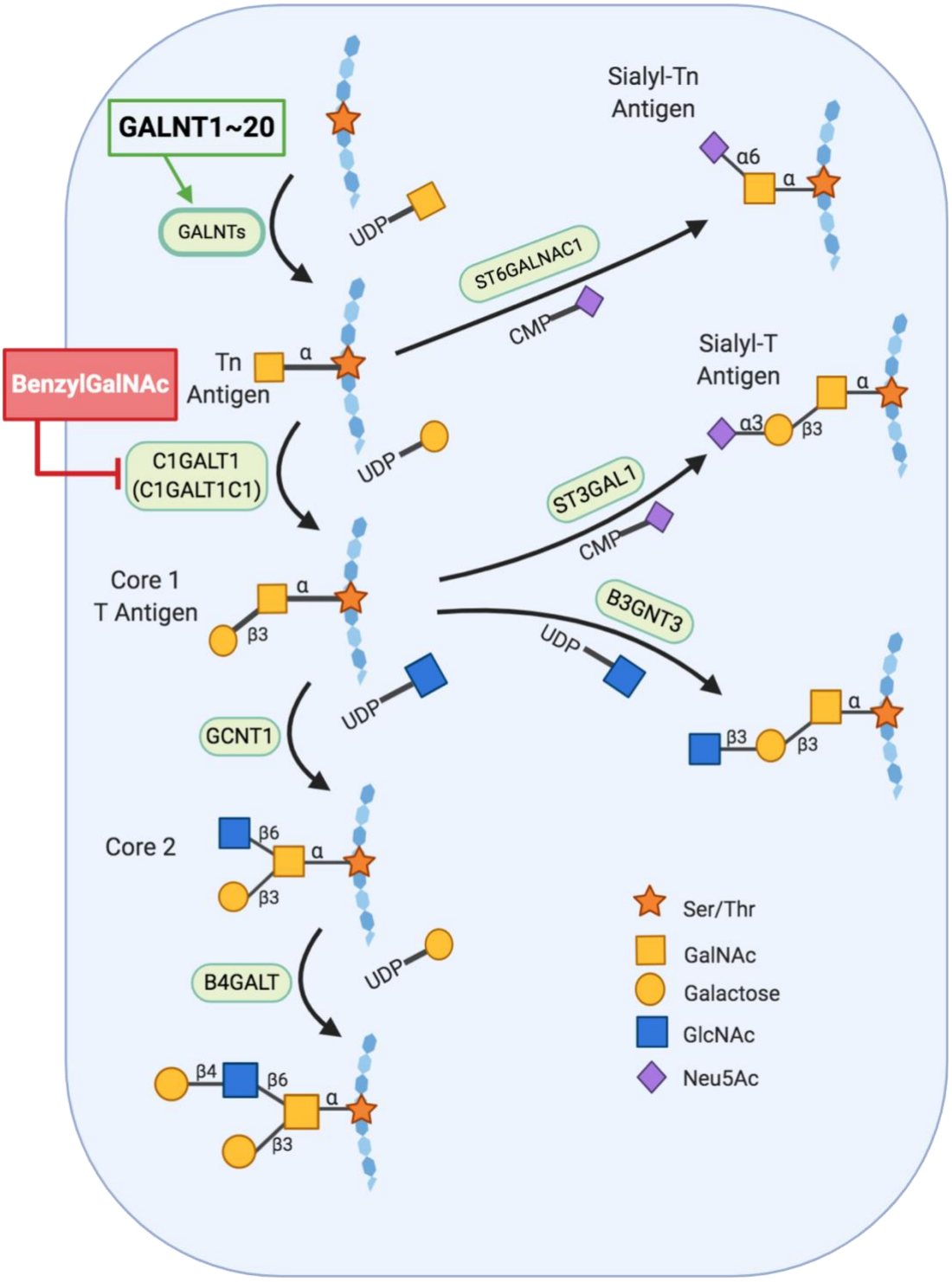


Fig. 6. A series of glycosylation of mucin O-glycan in the Golgi apparatus. Glycosylation enzymes including GALNT5, GALNT8, and GALNT18 (in bold) were induced upon BenzylGalNAc treatment, which are discussed in section 3.2.

Association of mucin-type O-glycans with many diseases has been studied robustly, though the significance of its biological function has been still poorly understood. Alteration of these O-glycosylation gives rise to diverse effects in animal

models varying from embryonic death to developmental defects and diseases. Inhibition of O-glycosylation either by mutation or other factors has been known to be linked with various effects and diseases. For instance, mutation of GALNT3 contributes to familial tumoral calcinosis (Ichikawa *et al.*, 2007; Kato *et al.*, 2006), while malfunction of the glycosylating enzyme T synthase causes Tn syndrome, leading to IgA nephropathy (Allen *et al.*, 2001; Hiki *et al.*, 1999, Hiki *et al.*, 1998). High-density lipoprotein metabolism impacts on coronary artery disease (Kathiresan *et al.*, 2008; Willer *et al.*, 2008), and tumor generation and metastasis instigation (Kim and Varki, 1997; Ono and Hakomori, 2003; Springer, 1997). Furthermore, it has been implicated with altered immune response, resulting in decreased rolling of leukocytes due to reduced adhesiveness of P-selectins, E-selectins, and L-selectins lacking mucin-type glycosylation (Sperandio, 2006).

BenzylGalNAc (BG) is a classic and well-known inhibitor of mucin-type O-glycosylation in the Golgi, and C1GALT1 is one of the key initiation enzymes in mucin-type O-glycosylation that can be inhibited by BenzylGalNAc as shown in Fig. 7 (Huet *et al.*, 1998; Kuans *et al.*, 1989). Thus, we speculated that if mucin-producing cells are treated with BG, the capacity of mucin glycosylation becomes insufficient, resulting in accumulation of less glycosylated mucin core proteins in the Golgi (mucin-type Golgi stress), and that cells may activate an unknown response pathway of the Golgi stress response to upregulate the expression of glycosylation enzymes for mucins, namely the mucin pathway. In this work, we have analyzed further whether mammalian cells have the mucin pathway of the Golgi stress response.

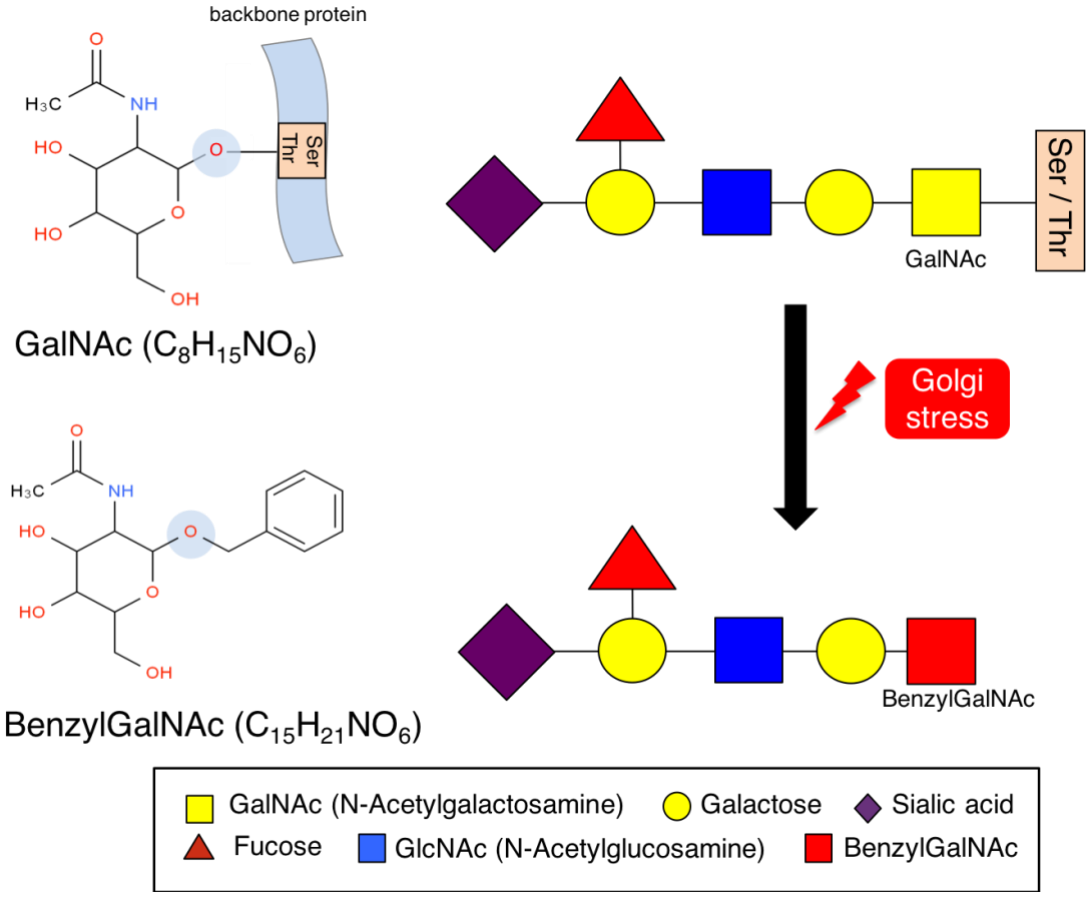


Fig. 7. BenzylGalNAc competitively inhibits O-glycosylation

CHAPTER TWO
MATERIALS AND METHOD

2.1 Cell Culture and Transfection

HT29 (human colon adenocarcinoma) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and kept in a humidified atmosphere of 5% CO2 at 37°C (Yoshida *et al.,* 2009). The calcium phosphate or PEI max methods was employed for transfection of plasmid DNAs into cells, treated with 10 mM BG for 48 h, washed with PBS and subjected to luciferase assays, immunoblotting or immunofluorescence microscopy (Yoshida *et al.,* 2006).

2.2 Construction of Plasmids

Corresponding regions of the human TFE3 promoter were amplified from human genomic DNA and cloned into the BglII site upstream of the firefly luciferase gene in a pGL4 basic vector (Promega, Fitchburg WI). The isolated GASE sequence from the human SIAT4A gene (a target gene of the TFE3 pathway) was inserted into the XhoI-BglII sites of pGL3-promoter vector (Promega) (Oku *et al.,* 2011). Point mutants of the promoter were made by site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene, CA) (Yoshida *et al.,* 2001).

Nucleotide sequences of oligo primer pairs used for construction of corresponding regions of the human TFE3 promoter are listed as follows:

For TFE3 promoter analysis,
-2017 ~ +291
Fw 5' GGAAGATCTAGACAGGGTTTTGCCACGTT 3'
Rv 5' GGAAGATCTGACGCCCGGCCCCGGGCGAG 3'
-1377 ~ +291
Fw 5' GGAAGATCTGTAGTTGGGATTACAGGCGC 3'

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-1048 ~ +291

Fw 5’ GGAAGATCTGCGCAACTCAGGGTGGGGTC 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-718 ~ +291

Fw 5’ GGAAGATCTGGAGGTCCCCTTCCCTATTC 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-383 ~ +291

Fw 5’ GGAAGATCTGCCGAAAGAGGGCGCGGTTC 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-60 ~ +291

Fw 5’ GGAAGATCTGATTGGCCGCCTCGCTAATG 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-49 ~ +291

Fw 5’ GGAAGATCTTCGCTAATGGGTCGTGGCCA 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-37 ~ +291

Fw 5’ GGAAGATCTCGTGGCCAGACGGGCCATCC 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-25 ~ +291

Fw 5’ GGAAGATCTGGCCATCCATCCCCAGTGCG 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-12 ~ +291

Fw 5’ GGAAGATCTCAGTGCGGTCACTTCCTGTG 3’

Rv 5’ GGAAGATCTGACGCCCGGCCCCGGGCGAG 3’

-12 ~ +239

Fw 5’ GGAAGATCTCAGTGCGGTCACTTCCTGTG 3’

Rv 5’ GGAAGATCTCCCCTAACAAAATAAGAGTC 3’

-12 ~ +179

Fw 5’ GGAAGATCTCAGTGCGGTCACTTCCTGTG 3’

Rv 5’ GGAAGATCTGCCATGGAGCTAGCACTGCG 3’

-12 ~ +119

Fw 5’ GGAAGATCTCAGTGCGGTCACTTCCTGTG 3’

Rv 5’ GGAAGATCTATCCCCCGCCCAGAACGGAC 3’

-12 ~ +59

Fw 5’ GGAAGATCTCAGTGCGGTCACTTCCTGTG 3’

Rv 5’ GGAAGATCTTCCCCCTCGTCTTCTTCCCC 3’

For TFE3 point mutation analysis,

wt (-12 ~ +19)

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCGCACTGG 3’

-12C>A

Fw 5’ GATCCAAGTGCGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCGCACTTG 3’

-11A>C

Fw 5’ GATCCCCGTGCGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCGCACGGG 3’

-10G>T

Fw 5’ GATCCCATTGCGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCGCAATGG 3’

-9T>G

Fw 5’ GATCCCAGGGCGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCGCCCTGG 3’

-8G>T

Fw 5’ GATCCCAGTTCGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCGAACTGG 3’

-7C>A

Fw 5’ GATCCCAGTGAGGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACCTCACTGG 3’

-6G>T

Fw 5’ GATCCCAGTGCTGTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGACAGCACTGG 3’

-5G>T

Fw 5’ GATCCCAGTGCGTTCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGAACGCACTGG 3’

-4T>G

Fw 5’ GATCCCAGTGCGGGCACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTGCCCGCACTGG 3’

-3C>A

Fw 5’ GATCCCAGTGCGGTAACTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGTTACCGCACTGG 3’

-2A>C

Fw 5’ GATCCCAGTGCGGTCCCTTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAAGGGACCGCACTGG 3’

-1C>A

Fw 5’ GATCCCAGTGCGGTCAATTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGAATTGACCGCACTGG 3’

+1T>G

Fw 5’ GATCCCAGTGCGGTCACGTCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGACGTGACCGCACTGG 3’

+2T>G

Fw 5’ GATCCCAGTGCGGTCACTGCCTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGGCAGTGACCGCACTGG 3’

+3C>A

Fw 5’ GATCCCAGTGCGGTCACTTACTGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACAGTAAGTGACCGCACTGG 3’

+4C>A

Fw 5’ GATCCCAGTGCGGTCACTTCATGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACATGAAGTGACCGCACTGG 3’

+5T>G

Fw 5’ GATCCCAGTGCGGTCACTTCCGGTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCACCGGAAGTGACCGCACTGG 3’

+6G>T

Fw 5’ GATCCCAGTGCGGTCACTTCCTTTGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCAAAGGAAGTGACCGCACTGG 3’

+7T>G

Fw 5’ GATCCCAGTGCGGTCACTTCCTGGGGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCCCCAGGAAGTGACCGCACTGG 3’

+8G>T

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTTGAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTCAACAGGAAGTGACCGCACTGG 3’

+9G>T

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGTAGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACTACACAGGAAGTGACCGCACTGG 3’

+10A>C

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGCGTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAACGCCACAGGAAGTGACCGCACTGG 3’

+11G>T

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGATTTTCCCCAA 3’

Rv 5’ GATCTTGGGGAAAATCCACAGGAAGTGACCGCACTGG 3’

+12T>G

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGGTTCCCCAA 3’

Rv 5’ GATCTTGGGGAACCTCCACAGGAAGTGACCGCACTGG 3’

+13T>G

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTGTCCCCAA 3’

Rv 5’ GATCTTGGGGACACTCCACAGGAAGTGACCGCACTGG 3’

+14T>G

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTGCCCCAA 3’

Rv 5’ GATCTTGGGGCAACTCCACAGGAAGTGACCGCACTGG 3’

+15C>A

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTTACCCAA 3’

Rv 5’ GATCTTGGGTAAACTCCACAGGAAGTGACCGCACTGG 3’

+16C>A

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTTCACCAA 3’

Rv 5’ GATCTTGGTGAAACTCCACAGGAAGTGACCGCACTGG 3’

+17C>A

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTTCCACAA 3’

Rv 5’ GATCTTGTGGAAACTCCACAGGAAGTGACCGCACTGG 3’

+18C>A

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTTCCCAAA 3’

Rv 5’ GATCTTTGGGAAACTCCACAGGAAGTGACCGCACTGG 3’

+19A>C

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGAGTTTCCCCCA 3’

Rv 5’ GATCTGGGGGAAACTCCACAGGAAGTGACCGCACTGG 3’

For MGSE motif mutation analysis,

Mutation of ‘ACTTCC’ motif

Fw 5’ GATCTCAGTGCGGTCCAGGAATGTGGAGTTTCCCCA 3’

Rv 5’ TGGGGAAACTCCACATTCCTGGACCGCACTGAGATC 3’

Mutation of ‘TCCCCA’ motif

Fw 5’ ACTTCCTGTGGAGTTGAAAACTCCCTGGGAGGAGGG 3’

Rv 5’ CCCTCCTCCCAGGGAGTTTTCAACTCCACAGGAAGT 3’

Mutation of both motif

Fw 5’ GATCTCAGTGCGGTCCAGGAATGTGGAGTTGAAAAC 3’

Rv 5’ GTTTTCAACTCCACATTCCTGGACCGCACTGAGATC 3’

For MGSE spacer mutation,

Fw 5’ GATCCCAGTGCGGTCACTTCCGTGTTCTGGTCCCCAA 3’

Rv 5’ GATCTTGGGGACCAGAACACGGAAGTGACCGCACTGG 3’

For MGSE spacer serial deletion mutation,

3 nt deletion

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTGGATCCCCAA 3’

Rv 5’ GATCTTGGGGATCCACAGGAAGTGACCGCACTGG 3’

6 nt deletion

Fw 5’ GATCCCAGTGCGGTCACTTCCTGTTCCCCAA 3’

Rv 5’ GATCTTGGGGAACAGGAAGTGACCGCACTGG 3’

9 nt deletion

Fw 5’ GATCCCAGTGCGGTCACTTCCTCCCCAA 3’

Rv 5’ GATCTTGGGGAGGAAGTGACCGCACTGG 3’

2.3 Quantitative RT-PCR

Total RNA was isolated from HT29 cells treated with appropriate Golgi stress inducers (benzylGalNAc or monensin) using Sepasol-RNA I Super G (Nacalai Tesque), in accordance with manufacturer’s protocol. Applied Biosystems™ 7500 Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA) and PrimeScript RT reagent kit with gDNA Eraser and SYBR Premix Ex Taq II (Tli RNase H Plus) (TaKaRa, Otsu, Japan)

were utilized to carry out quantitative RT-PCR (qRT-PCR) (Sasaki *et al.*, 2019). Nucleotide sequences of primer pairs used here were as follows:

GAPDH
Fw 5’ GGCACAGTCAAGGCTGAGAATG 3’
Rv 5’ ATGGTGGTGAAGACGCCAGTA 3’
TFE3
Fw 5’ ACTGGGCACTCTCATCCCTAAGTC 3’
Rv 5’ TTCAGGATGGTGCCCTTGTTC 3’
HSP47
Fw 5’ AAGAGCAGCTGAAGATCTGGATG 3’
Rv 5’ GTCGGCCTTGTTCTTGTCAATG 3’
GALNT5,
Fw 5’ GGCCTGTCCAGTAATCGAAGTCA 3’
Rv 5’ AAAGTTCATGGGCCACACAAAGA 3’
GALNT8
Fw 5’ AACCATGCTCCAAGGCAGCTA 3’
Rv 5’ CATCTCCAGACACCGCTTGGTA 3’
GALNT18
Fw 5’ TGCCTGACCTCAGACCCCAGT 3’
Rv 5’ TGTCATCCACCAGAATGATCTCC 3’

2.4 Luciferase Assay

Transient transfection of HT29 cells was carried out as described in section 2.1. HT29 cells cultured in 24-well plates or 96-well plates were harvested with passive lysis buffer (ToyoInk, Tokyo, Japan). Activities of Firefly and Renilla luciferase were measured with 7.5 μl of cell lysates using the PicaGene Dual Sea Pansy Luminescence Kit (ToyoInk, Tokyo, Japan) and measured by CentroXS LB960 luminometer (Berthold, Wildbad, Germany). Relative activity was defined as the ratio of firefly luciferase activity to Renilla luciferase activity and was calculated by simply dividing luminescence intensity obtained with the assay for firefly luciferase by that for Renilla luciferase.

2.5 Immunoblotting

Whole cell extracts were prepared by lysing HT29 cells with 1x 40 μl sample buffer (sample buffer contain 20 μl of 4xSDS [200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue and 40% glycerol] and 20 μl of PBS:Protease inhibitor:Phospatase inhibitor of 100:40:1 ratio, respectively). Lysates were boiled, and portions (8 μl) were subjected to SDS-polyacrylamide gel electrophoresis using 10% gel, transferred onto a Immobilon-P transfer membrane (MilliporeSigma, USA), and reacted with anti-TFE3, anti-β-tubulin (primary antibody), or anti-rabbit IgG-HRP (GE Health care) (secondary antibody) antisera according to the standard protocol. Anti-TFE3 antiserum was prepared in the previous study (Taniguchi et al., 2015). Prestained SDS-PAGE standards (Bio-rad) were used as size markers. The EZ-ECL blotting detection kit (Biological Industries) was used to detect each antigen.

2.6 Immunocytochemistry

HeLa or HT29 cells grown on cover slips were transiently transfected with an appropriate expression plasmid by the calcium phosphate method. Cells were fixed with 4% paraformaldehyde/PBS for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, and stained with appropriate antibody (anti-Giantin, anti-GM 130 or anti-TFE3). Anti-Giantin and anti-GM130 antisera were purchased from COVANCE (Princeton, NJ) (#PRB-114C) and Becton Dickinson and Company (Franklin Lakes, New Jersey) (#610823), respectively. Anti-TFE3 antiserum was prepared in the previous study (Taniguchi *et al.*, 2015). Cover slips were mounted with 5 μl SlowFade Gold Antifade Reagents (Life technologies) containing 0.2 ng/μl of DAPI. Images were acquired using a N*i*-U microscope (Nikon) and ORCA-Flash4.0 V3 digital CCD camera (Hamamatsu Photonics).

2.7 Microarray Analyses and RNA Sequencing with a Next-Generation DNA Sequencer

Total RNA was extracted from HT29 cells treated with 10 mM BG for 48 h by the acid guanidium/phenol/chloroform method using Sepasol (Nacalai Tesque) and purified

using RNeasy Mini column (Qiagen). Microarray analyses were performed using the SurePrint G3 Human GE 8x60K v2 Microarray (Agilent Technologies, Santa Clara, CA) in collaboration with TaKaRa Bio Inc. RNA-Seq analyses were performed with the Ion Proton System for Next-Generation Sequencing with PI chips (Thermo Fisher Scientific, Waltham, MA). The microarray and RNA-seq data were deposited in GEO database (GEO accession numbers are GSE117938 and GSE119909, respectively).

CHAPTER THREE
RESULTS

3.1 Insufficiency of Mucin-Type O-glycosylation (Golgi stress) Resulted in Fragmentation of the Golgi apparatus

Mucins are glycoproteins that receive a number of mucin-type O-glycans in the Golgi apparatus by specific glycosylation enzymes for mucins such as GALNTs (Jensen *et al.*, 2010). Thus, it is possible that the capacity of mucin-type glycosylation becomes insufficient, and expression of these glycosylation enzymes should be induced by the mucin pathway of the Golgi stress response when cells increase expression of mucins. Actually, it has been reported that the Golgi apparatus becomes well developed during differentiation of mucin-secreting cells in the Brunner's glands (Fig. 2B) (Berger and Roth, 1997). The main theme of this thesis is to reveal the molecular mechanism of the mucin pathway. To investigate the mucin pathway, I developed two types of the experimental systems in which the mucin pathway is artificially activated in culture cells, that is, overexpression of mucin core proteins and inhibition of mucin-type glycosylation by treatment of BenzylGalNAc (BG). I speculated that overexpression of mucin core proteins requires large number of glycosylation enzymes and this may cause insufficiency of glycosylation enzymes for mucins in the Golgi (mucin-type Golgi stress), resulting in activation of the mucin pathway. As mentioned in the literatures of Chapter 1.4, BG is a chemical compound that inhibits mucin-type O-glycosylation, and could cause insufficiency of mucin type O-glycosylation (mucin-type Golgi stress), resulting in activation of the mucin pathway.

First, I examined the effect of overexpression of mucin core proteins such as MUC1 and MUC20 on the morphology of the Golgi in HeLa cells. HeLa cells were used for this experiment instead of HT29 cells, because HeLa cells synthesize lower amount of mucins and the effect of overexpression of mucin core proteins in HeLa cells seemed greater than that in HT29 cells, which synthesize relatively large amount of mucins. HeLa cells were transfected with an expression vector of MUC1 or MUC20 (3xFLAG-MUC1 or 3xFLAG-MUC20) for 48 hours and were stained using anti-Giantin (the *cis* and *medial*-Golgi marker) and anti-FLAG antisera for

immunofluorescence observation as described in “Experimental Procedures” section in Chapter 2.6. The Golgi apparatus in HeLa cells that were not transfected with an expression vector showed typical Golgi morphology (indicated by open arrow heads in Fig. 8A and Fig. 8B), whereas, the Golgi in HeLa cells transfected with an expression plasmid of 3xFLAG-MUC1 or 3xFLAG MUC20 was severely fragmented, suggesting that the functional capacity of Golgi was overwhelmed and leading to the fragmentation of Golgi (closed arrow heads in Fig. 8A and Fig. 8B).

Next, I examined the effect of BG on Golgi morphology. HT29 cells were treated with BG for 48 hours and was assessed by immunofluorescence microscopy. Immunofluorescence staining for the *cis*-Golgi marker GM130 revealed that the Golgi was substantially disassembled when cells were treated with BG whereas (Fig. 8C, panels E and F), whereas the Golgi was intact in cells treated with DMSO (panels B and C), suggesting that BG could be used to activate the mucin pathway. We then repeated the same experiment with HeLa cells and found that the disruption of Golgi in HeLa cells was less severe as compared to the disruption of Golgi in HT29 cells (Fig. 8D, panels E and F). It is important to note that HeLa cells produce lower amount of mucin proteins, suggesting that the disruption of Golgi induced by BG is dependent with the amount of mucin production. Presumably, proteins incompletely modified with mucin-type glycans might accumulate in the Golgi and cause disruption of Golgi morphology.

From these observations that inhibition of glycosylation by BG and overexpression of mucin core protein overwhelms the Golgi functional capacity and induces Golgi stress, we concluded that the mucin pathway of the Golgi stress response does exist and mucin-type Golgi stress causes perturbation of the Golgi, leading to fragmentation of Golgi morphology.

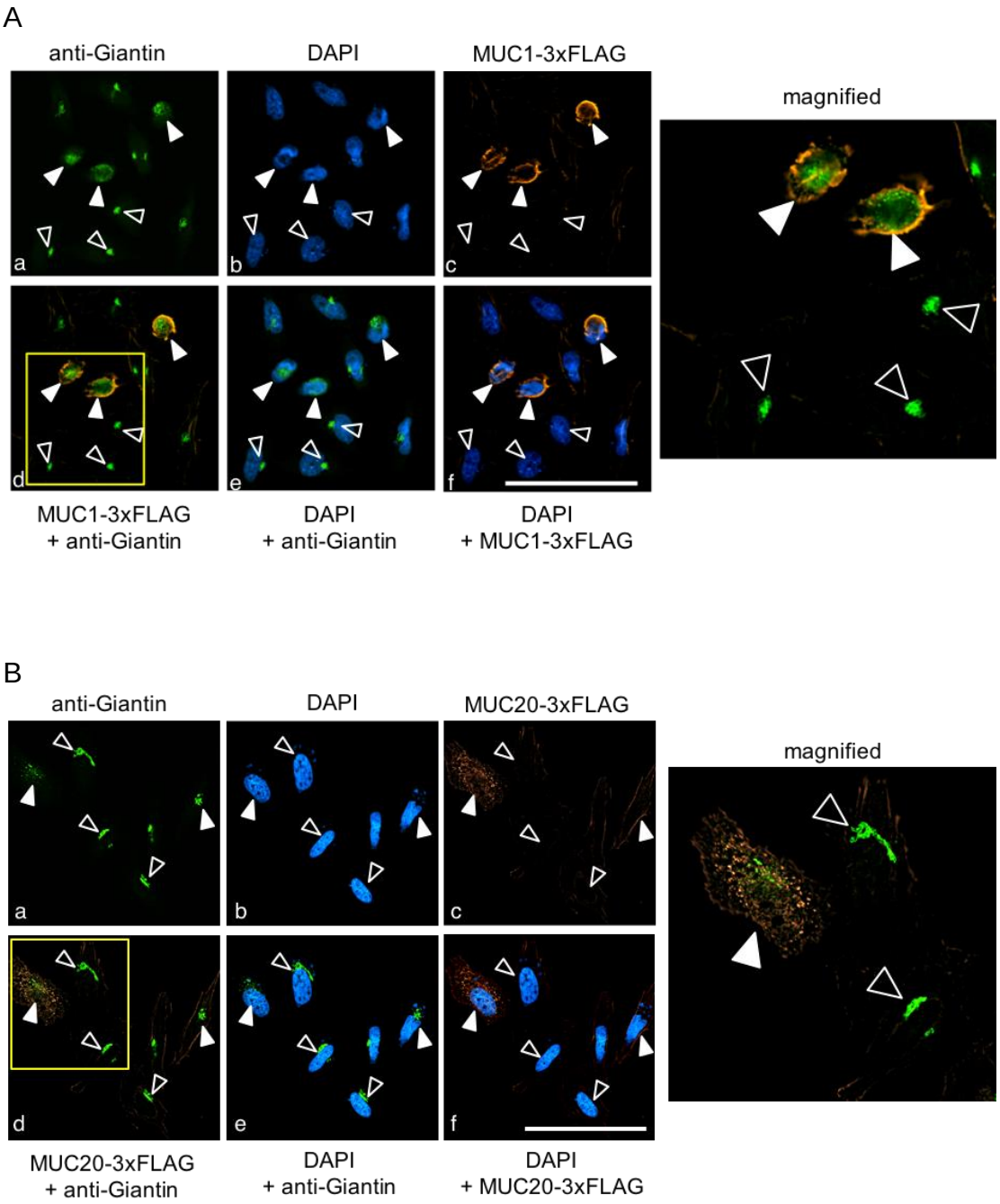
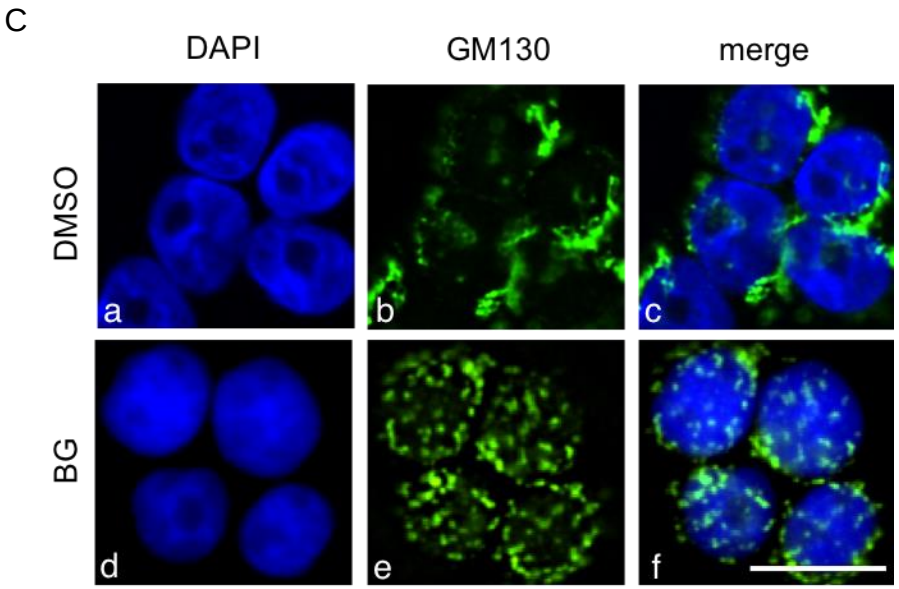
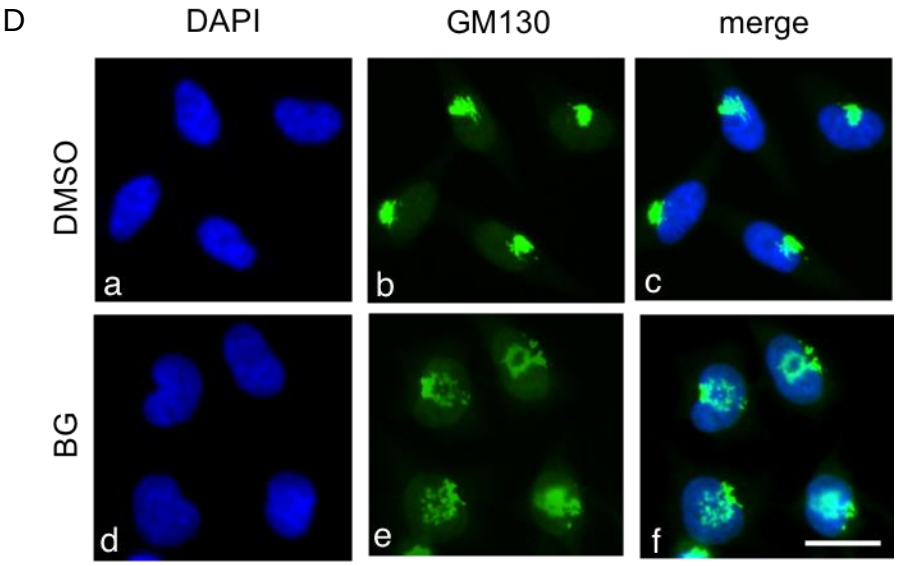


Fig. 8 Morphological changes of the Golgi apparatus upon mucin type-Golgi stress. (A) HeLa cells transfected with a 3×FLAG-MUC1 expression vector were stained with anti-FLAG (orange) and anti-Giantin (green) antisera. Bars = 50 μm. Closed and open arrow heads indicate cells that overexpressed or did not overexpress 3×FLAG-MUC1, respectively. (B) HeLa cells transfected with a 3×FLAG-MUC20 expression vector were stained with anti-FLAG (orange) and anti-Giantin (green) antisera. Bars = 50 μm. Closed and open arrow heads indicate cells that overexpressed and did not overexpress 3×FLAG-MUC20, respectively. (C) HT29 cells treated with 10 mM BG for 48 h were stained with DAPI (blue) and anti-GM130 antibody (green). Bars = 10 μm. (D) The same experiment as (C) was performed using HeLa cells.


C
DAPI
GM130
merge
DMSO
BG
a
b
c
d
e
f


D
DAPI
GM130
merge
DMSO
BG
a
b
c
d
e
f

(continued)

3.2 Mucin-type Golgi Stress Induces the Transcription of HSP47 Gene and Mucin-related Genes

It was previously reported that upon treatment with BG mucin-type O-glycosylation in the Golgi is severely inhibited and the level of HSP47 mRNA was increased in Colo 205 and NIH3T3 cells in order to suppress Golgi stress-induced cell apoptosis (Miyata *et al.*, 2013). Prompted by a previous study, we recapitulated HSP47 mRNA induction in HT29 cells using qRT-PCR to confirm that BG can induce mucin-type Golgi stress in HT29 cells. As shown in Fig. 9A, we found that the expression of HSP47 mRNA was elevated after BG treatment for 48 h (lanes 1 and 2). This finding indicates that BG induces Golgi stress and increases transcription of HSP47 mRNA in our experimental system.

To check whether BG treatment induces transcription of genes encoding mucin-type glycosylation enzymes, total RNA was prepared from HT29 cells treated with BG for 48 h and subjected to microarray analyses (data not shown) and qRT-PCR analyses. Akin to the effect of BG on the level of HSP47 mRNA, we found that the expression levels of GALNT5, GALNT8, and GALNT18 mRNA were also upregulated upon BG treatment (Fig. 9B-D). These findings strongly suggested that the expression of enzymes for mucin-type glycosylation was augmented by mucin-type Golgi stress (insufficiency of mucin glycosylation) upon BG treatment. Together with the results from section 3.1, these observations suggest that mucin-type Golgi stress was induced by inhibition of mucin-type O-glycosylation and named this novel response pathway as the mucin pathway.

3.3 Mucin-type Golgi Stress Unexpectedly Induced Transcription of Human TFE3 Gene

Transcription factor E3 (TFE3) is a basic-helix-loop-helix domain-containing transcription factor that is a key transcription factor regulating the TFE3 pathway of the mammalian Golgi stress response (Taniguchi *et al.*, 2015). In normally growing cells, TFE3 is highly phosphorylated and retained in the cytoplasm, whereas upon treatment with activators of the TFE3 pathway such as monensin and nigericin, TFE3 is activated by dephosphorylation at Ser108, although there is no upregulation of TFE3 mRNA. Surprisingly, we found that BG treatment increased the expression of TFE3 mRNA

(Fig. 9E). We then further examined whether BG can elevate the transcription of human TFE3 gene independently from the activation of the TFE3 pathway. When HT29 cells were treated with monensin (a typical inducer of the TFE3 pathway) and subjected to qRT-PCR, we found that expression of TFE3 mRNA was not affected by monensin treatment (Fig. 9F). Taken together, these results indicated that the mucin pathway induces TFE3 transcription independently of the TFE3 pathway, and that there is an interesting possibility of the existence of crosstalk from the mucin pathway to the TFE3 pathway in the mammalian Golgi stress response, which prompted us to devote our investigation on the molecular mechanism of the crosstalk between these signaling pathways for the rest of this study.

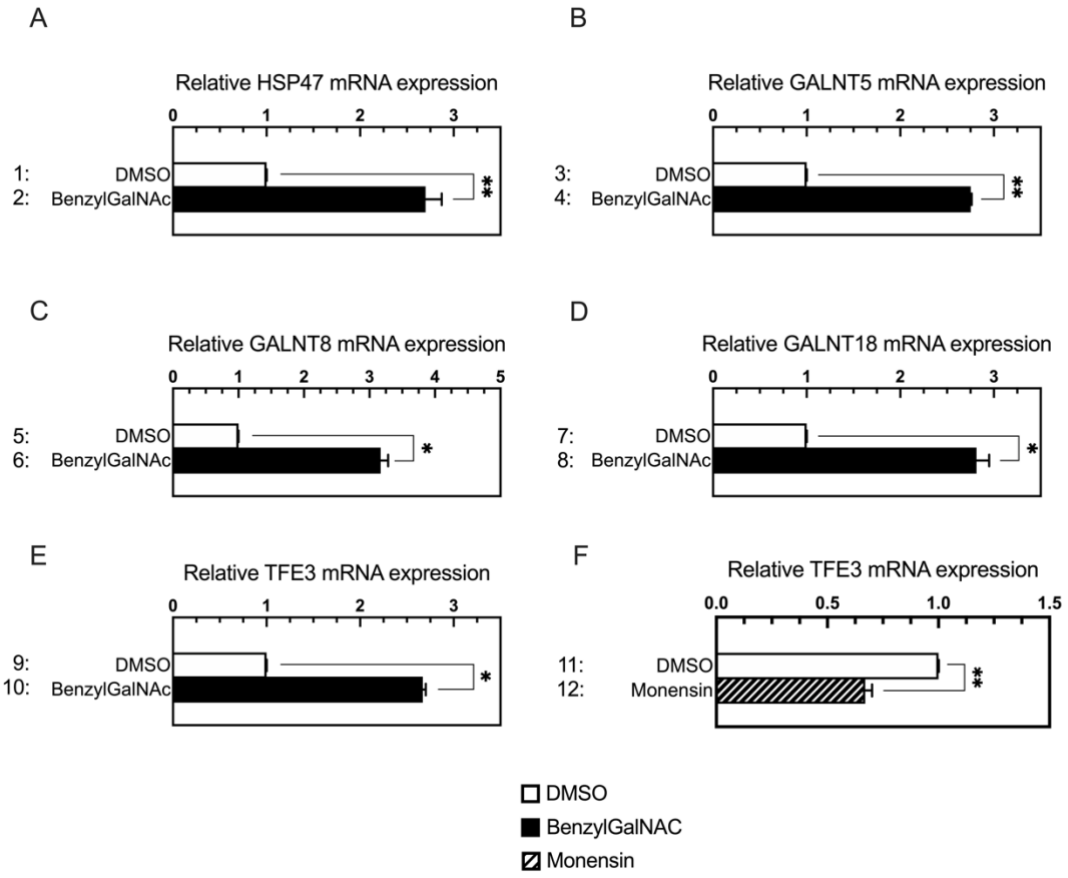


Fig. 9. Effect of BG on the expression of HSP47, GALNT8, GALNT5, GALNT18, and TFE3 mRNAs. Total RNA prepared from HT29 cells treated with 10 mM BG for 48 h or 0.3 μm monensin were subjected to qRT-PCR experiments (A, B, D, E, and F) or to RNA sequencing.

3.4 Analysis of human TFE3 Gene Promoter upon Mucin-type Golgi Stress

To clarify the molecular mechanism of the crosstalk from the mucin pathway to the TFE3 pathway, we performed promoter analysis to investigate the effect of BG treatment on transcription from the promoter of the human TFE3 gene. The [-2017 to +291] region of the human TFE3 gene (numbers indicate the position from the transcription start site) and its deletion constructs indicated in the Fig. 10 were fused with a firefly luciferase gene and transfected into HT29 cells. HT29 cells were then treated with BG for 48 h before the cells were harvested for luciferase assays (Fig. 10). Transcriptional induction by BG treatment was evaluated by measuring the luciferase activity of these constructs corrected by Renilla luciferase activity which was used as a standard of transfection efficiency. From these experiments, we found that all deletion constructs shown in Fig. 10 exhibited high activity of transcriptional induction upon BG treatment, suggesting that the [-12 to +19] region is sufficient for transcriptional induction of human TFE3 gene upon mucin-type Golgi stress, and that the enhancer element regulating transcriptional induction resides within that region.

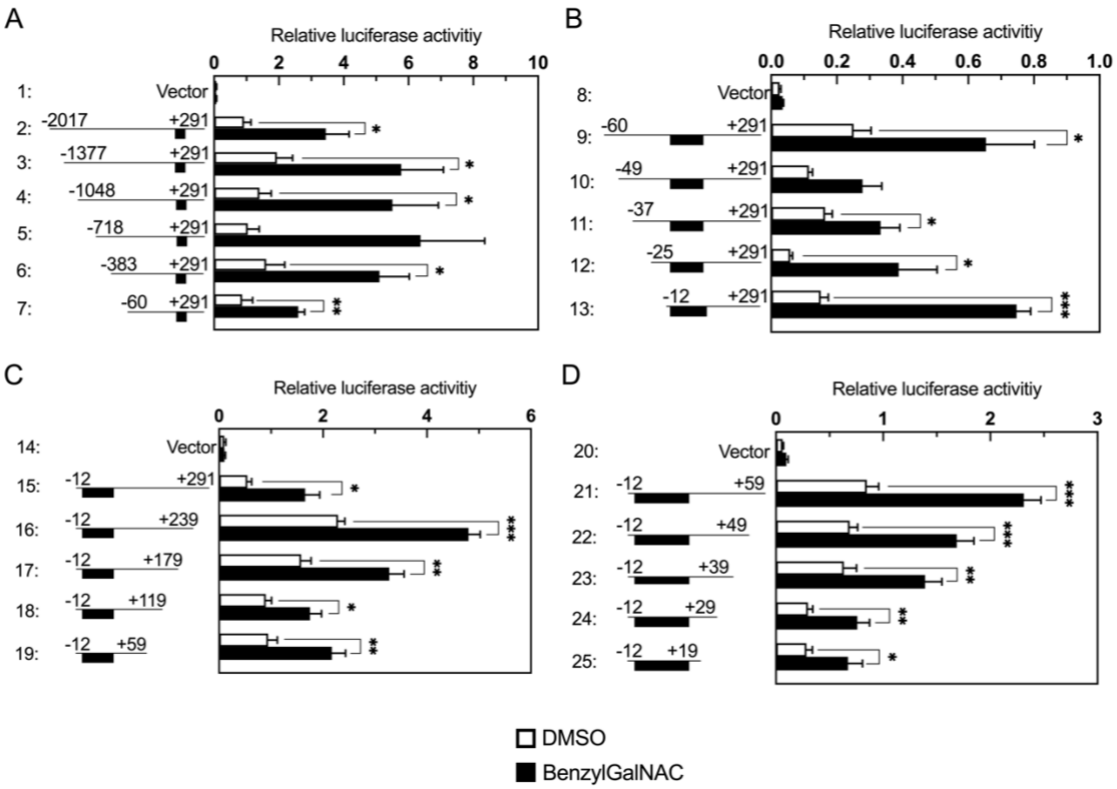


Fig. 10. Promoter analysis of the human TFE3 promoter. HT29 cells were transiently transfected with reporter constructs containing the indicated regions of the human TFE3 promoter and a luciferase reporter gene, treated with 10 mM BG for 48 h, and subjected to luciferase assays. The estimated locations of enhancers regulating transcriptional induction are indicated by black boxes. The vacant reporter plasmid pGL4 basic was used as control. Values are means ± SE of six independent experiments. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

3.5 Identification of an Enhancer Element Activating Transcriptional Induction of the Human TFE3 Gene

To determine the critical sequence of the enhancer element regulating the transcriptional induction of TFE3, we introduced point mutations into each nucleotide within the [-12 to +19] region, and the mutant promoters fused with luciferase gene were subjected to luciferase assays as described in Chapter 2.4 (Fig. 11). In contrast to the activity of transcriptional induction of a wild-type (WT) sequence (lane 2), those of point mutants in Region A or Region B were markedly reduced (lanes 13-18 and lanes 28-33, respectively). These results suggested that the critical sequence of the enhancer element activating transcription of human TFE3 gene is ACTTCC(N9)TCCCCA. We named this enhancer element as the mucin-type Golgi stress response element (MGSE).

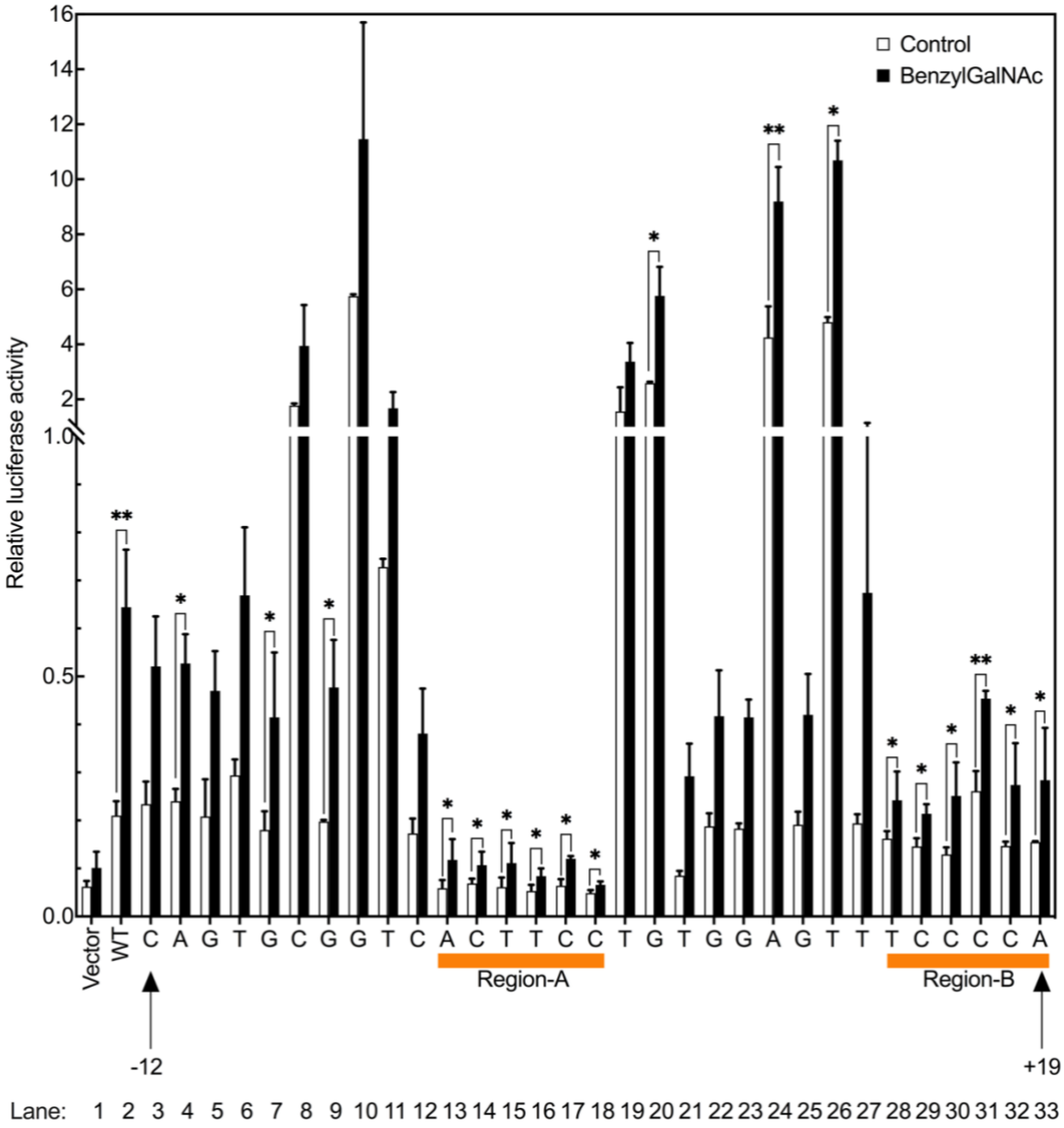


Fig. 11. Point mutation analysis of the [-12 to +19] region of the human TFE3 promoter. HT29 cells were transiently transfected with luciferase reporters containing the [-12 to +19] region of human TFE3 promoter, treated with 10 mM BG for 48 h, and subjected to luciferase assays. Each single nucleotide in the [-12 to +19] region was mutated to another nucleotide (A, T, G, and C were replaced by C, G, T, and A, respectively). The vacant reporter plasmid pGL4 basic was used as vector control. Values are means ± SE of five independent experiments.

3.6 The MGSE is Essential for Transcriptional Induction of TFE3 upon Mucin-type Golgi Stress

From the above results, we discovered that MGSE has two motifs separated by 9 nucleotides (nt). To reveal whether the two motifs of MGSE sequence and 9 nt of spacing between these two motifs is essential for transcriptional induction of the TFE3 promoter, we strategically constructed several mutant TFE3 promoters and evaluated them by luciferase assay as follows (Fig. 12). First, we evaluated the importance of Region A and Region B motifs by mutating Region A, Region B or both (Fig. 11A). A vector control was barely affected by BG treatment (lane 1), whereas a reporter containing the WT promoter ([-12 to +292]) significantly increased luciferase activity upon BG treatment (lane 2). Meanwhile, mutation of "ACTTCC", "TCCCCA" or both markedly reduced transcriptional induction compared to the wild type (lanes 3-5), implying that both motifs of MGSE are essential for activity of MGSE. Although it can be noted that the P-value of lane 1 in Fig. 12A has one asterisk, it appears that luciferase activity was too low, which leads to the fluctuation of the P-value.

Next, we evaluated the importance of the spacer sequence "TGTGGAGTT" that separates the "ACTTCC" and the "TCCCCA" motifs (Fig. 12B). We replaced the WT spacer sequence with "GTGTTCTGG" and measured the luciferase activity upon BG treatment together with the WT in lane 7 and 8. The luciferase activity of the mutated spacer sequence was not significantly different compared to the WT, suggesting that the nucleotide sequence of the spacer region itself is not essential for MGSE activity.

Finally, we evaluated the importance of the 9 nt distance between the "ACTTCC" and "TCCCCA" motifs (Fig. 12C). Mutant MGSEs containing only 6, 3, or 0 nt spacer sequences exhibited significantly reduced activity (lanes 11-13), indicating that the 9 nt distance that separates two motifs (but not the nucleotide sequence itself) is crucial for the activity of MGSE. These results suggested that ACTTCC(N9)TCCCCA is the critical sequence of MGSE and is sufficient for transcriptional induction of TFE3 in response to mucin-type Golgi stress.

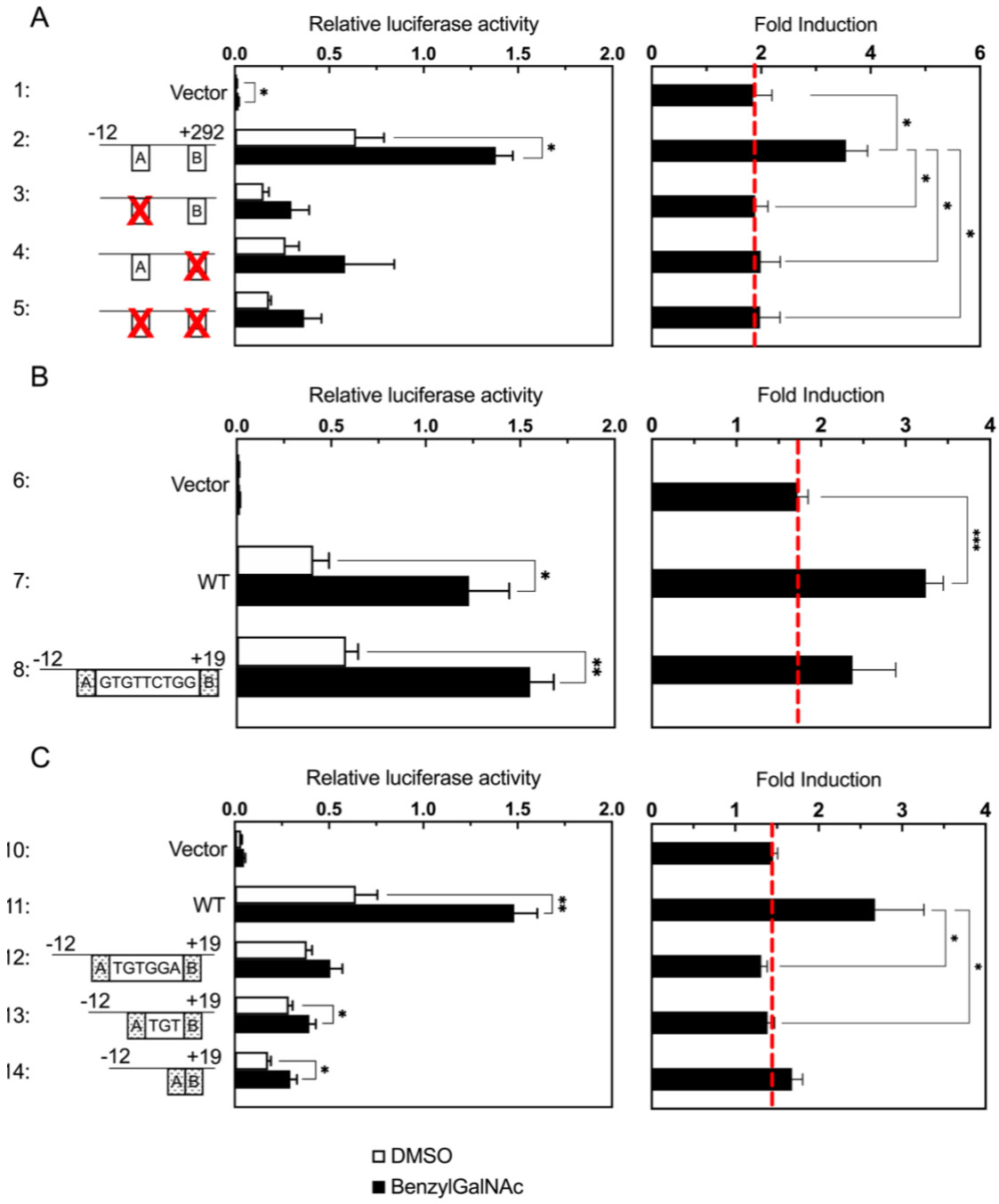


Fig. 12. Contribution of MGSE to transcriptional induction of the TFE3 gene and importance of the spacer region of MGSE. (A) Mutation of the MGSE sequence in the [-12 to +292] region of the human TFE3 promoter. HT29 cells transfected with the indicated reporter plasmids were treated with 10 mM BG for 48 h and subjected to luciferase assays. A and B indicate the ACTTCC and TCCCCA motifs of MGSE, respectively. (B) Mutation of the spacer sequence between ACTTCC and TCCCCA motifs of MGSE. The spacer sequence of wild-type 'TGTGGAGTT' was mutated to 'GTGTTCTGG'. Activity of the mutated sequences ([-12 to +19]) were evaluated as described in (A). (C) Stepwise deletion mutation of the spacer sequence. Cells transfected with the indicated reporter plasmids were processed as described in (B). Reporter plasmid pGL4 basic was used as the control. Values are means ± SE of three independent experiments. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

3.7 Transcription from MGSE is Activated by Overexpression of Mucin Core Proteins

In the section 3.1, we performed two experimental approaches to activate the mucin pathway, either by overexpression of mucin core proteins (MUC1 or MUC20) or inhibition of mucin-type O-glycosylation by BG. As we have identified the MGSE enhancer via induction by BG treatment only, it is possible that the transcriptional induction from MGSE by BG treatment is not related to mucin-type Golgi stress since BG is a small chemical compound. Therefore, in order to confirm that MGSE is essential to enhance transcription of TFE3 upon mucin-type Golgi stress, we investigated whether MUC1 or MUC20 overexpression can induce the transcription from MGSE (Fig. 13). We co-transfected HT29 cells with an expression vector of 3×FLAG-MUC1 or 3×FLAG-MUC20 as well as a 1× or 3×MGSE reporter, and measured relative luciferase activity. Overexpression of MUC1 or MUC20 had hardly affected luciferase activity of a vacant reporter (lanes 1 and 4), whereas it had significantly increased transcription from 1xMGSE or 3xMGSE (lanes 2, 3, 5, and 6), suggesting that transcription from MGSE is activated by the overexpression of mucin core proteins. These results strongly supported our notion that MGSE is an enhancer regulating transcriptional induction of TFE3 in response to mucin-type Golgi stress.

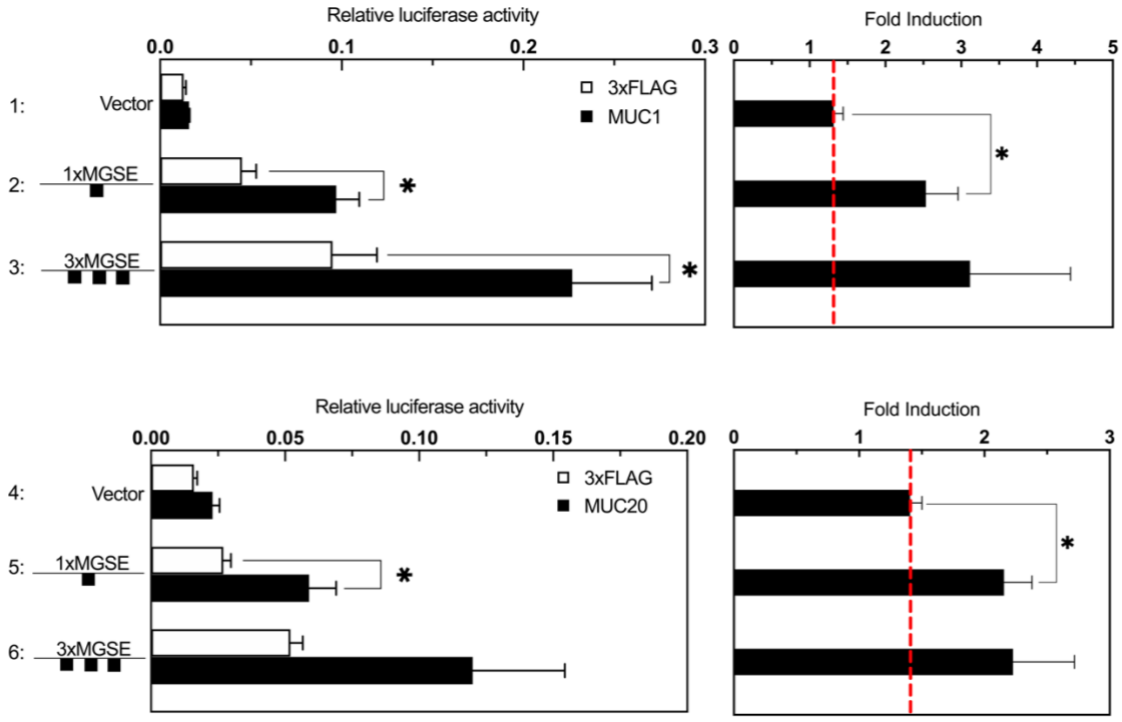


Fig. 13. HT29 cells transiently co-transfected with a 3×FLAG-MUC1 or 3×FLAG-MUC20 expression plasmid and a luciferase reporter containing 1×MGSE and 3×MGSE as indicated. A vacant reporter plasmid pGL4 basic was used as a control. Values are means ± SE of three independent experiments. *, $P < 0.05$.

3.8 Mucin-type Golgi Stress Induced TFE3 Dephosphorylation and Nuclear Translocation but Unable to Induce GASE

As mentioned in section 3.3, induction of TFE3 transcription by mucin-type Golgi stress has sparked an interesting possibility of existence of crosstalk by which mucin-type Golgi stress could activate not only the mucin pathway but also the TFE3 pathway. In normally growing cells, TFE3 is retained in the cytoplasm as a dormant form through phosphorylation at Ser108. Upon activation of the TFE3 pathway, three main events occur; (1) dephosphorylation of TFE3 at Ser108, (2) nuclear translocation of TFE3 after dephosphorylation, and subsequently, (3) transcriptional induction of target genes of the TFE3 pathway (such as GCP60, SIAT4A and SIAT10) from an enhancer element GASE. Hence, we sought to examine the crosstalk mechanism by analyzing the activation status of the TFE3 pathway upon mucin-type Golgi stress, based on these three events.

First, we examined the phosphorylation status of TFE3 upon mucin-type Golgi stress by immunoblotting (Fig. 14). In line with previous report (Taniguchi *et al.*, 2015), we observed eight bands of TFE3 in HT29 cells in normally growing cells as shown in Fig. 14A (lane 1 and lane 5), The four upper bands correspond to the large forms of TFE3 (TFE3(L)), and the four lower bands are derived from the short forms (TFE3(S)). The difference in molecular weight of each of the four bands is due to differences in phosphorylation status. When HT29 cells were treated with vehicle (DMSO), the phosphorylation status of TFE3 barely changed (lanes 2–4). On the other hand, we found that TFE3 was started to dephosphorylate around 4 h (lane 6) and later, more dephosphorylation of TFE3 was detected around 8 h after BG treatment (lanes 7 and 8), suggesting that mucin-type Golgi stress induces TFE3 dephosphorylation. Expression of TFE3 was increased 8 h after BG treatment, reflecting transcriptional induction of TFE3 by mucin-type Golgi stress.

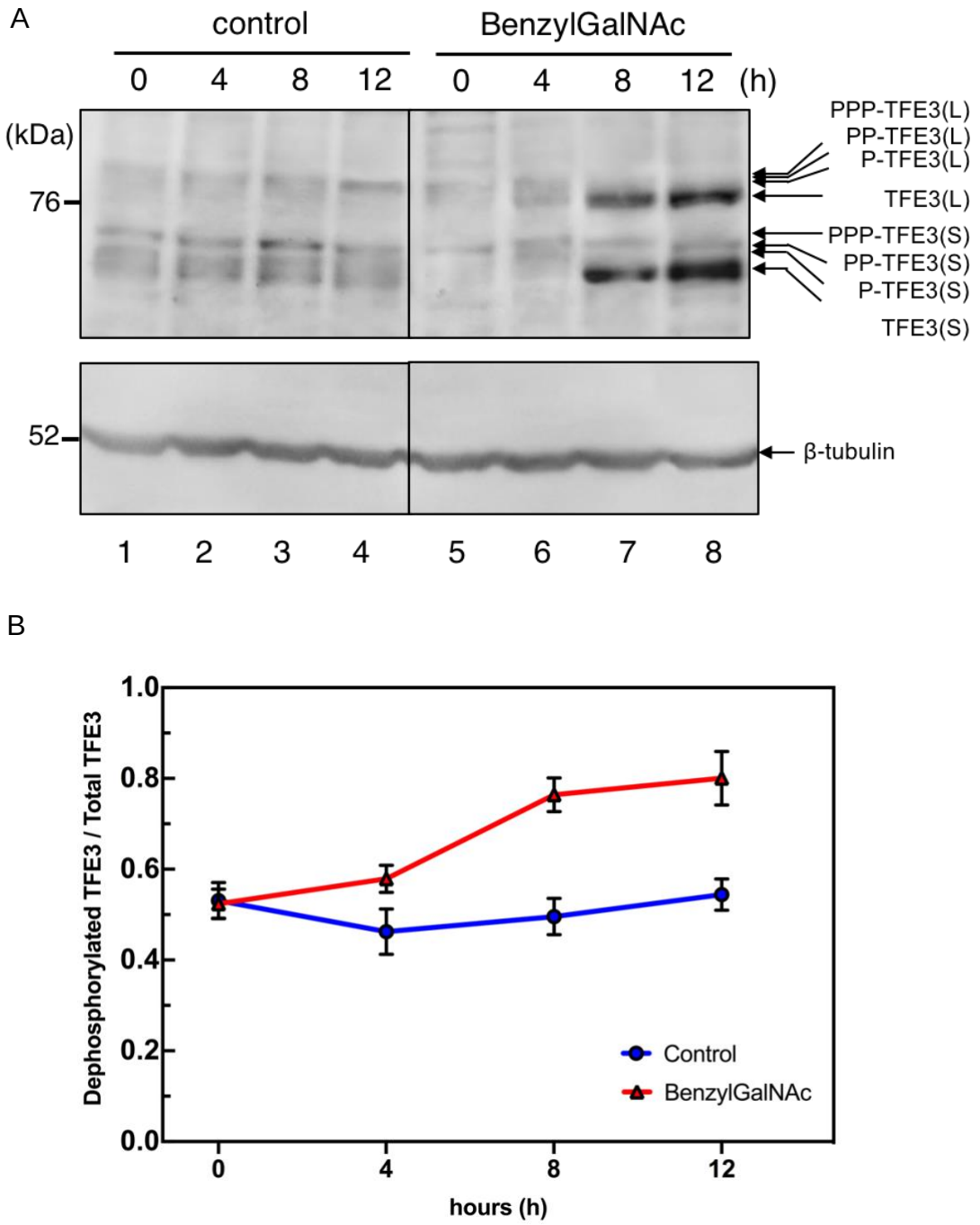


Fig. 14. Phosphorylation status of TFE3 upon BG treatment. (A) HT29 cells treated with 10 mM BG for the indicated time periods were subjected to immunoblotting using anti-TFE3 and anti-β-tubulin antisera. TFE3(L) and (S) are long and short forms of TFE3, respectively, and (P-) represents phosphorylated TFE3. (B) Ratio of signal detected between dephosphorylated TFE3 and total TFE3. Signals of TFE3(L) and (S) in (B) were quantified and plotted accordingly.

Second, we investigated whether TFE3 translocates into the nucleus upon BG treatment (Fig. 15). When endogenous TFE3 was stained with anti-TFE3-A antiserum in normally growing HT29 cells, TFE3 was observed in the cytoplasm (panels a–c). Interestingly, after BG treatment, TFE3 was localized in the nucleus (panels d–r), demonstrating that TFE3 rapidly translocates into the nucleus upon mucin-type Golgi stress. Unexpectedly, TFE3 in BG-treated cells was stained as particles in the nucleus, and we speculate that TFE3 may be located on the promoters of its target genes or that TFE3 formed liquid droplets by liquid–liquid phase separation. We then further examined whether overexpression of MUC1 or MUC20 causes nuclear translocation of TFE3, and found that TFE3 was localized in the nucleus in cells overexpressing MUC1 or MUC20 (closed arrowhead) (Fig. 16A and Fig. 16B). On the contrary, TFE3 remained in the cytoplasm in cells that did not overexpress 3×FLAG-MUC1 and 3×FLAG-MUC20, respectively (open arrowhead). These results suggest that mucin-type Golgi stress induces nuclear translocation of TFE3.

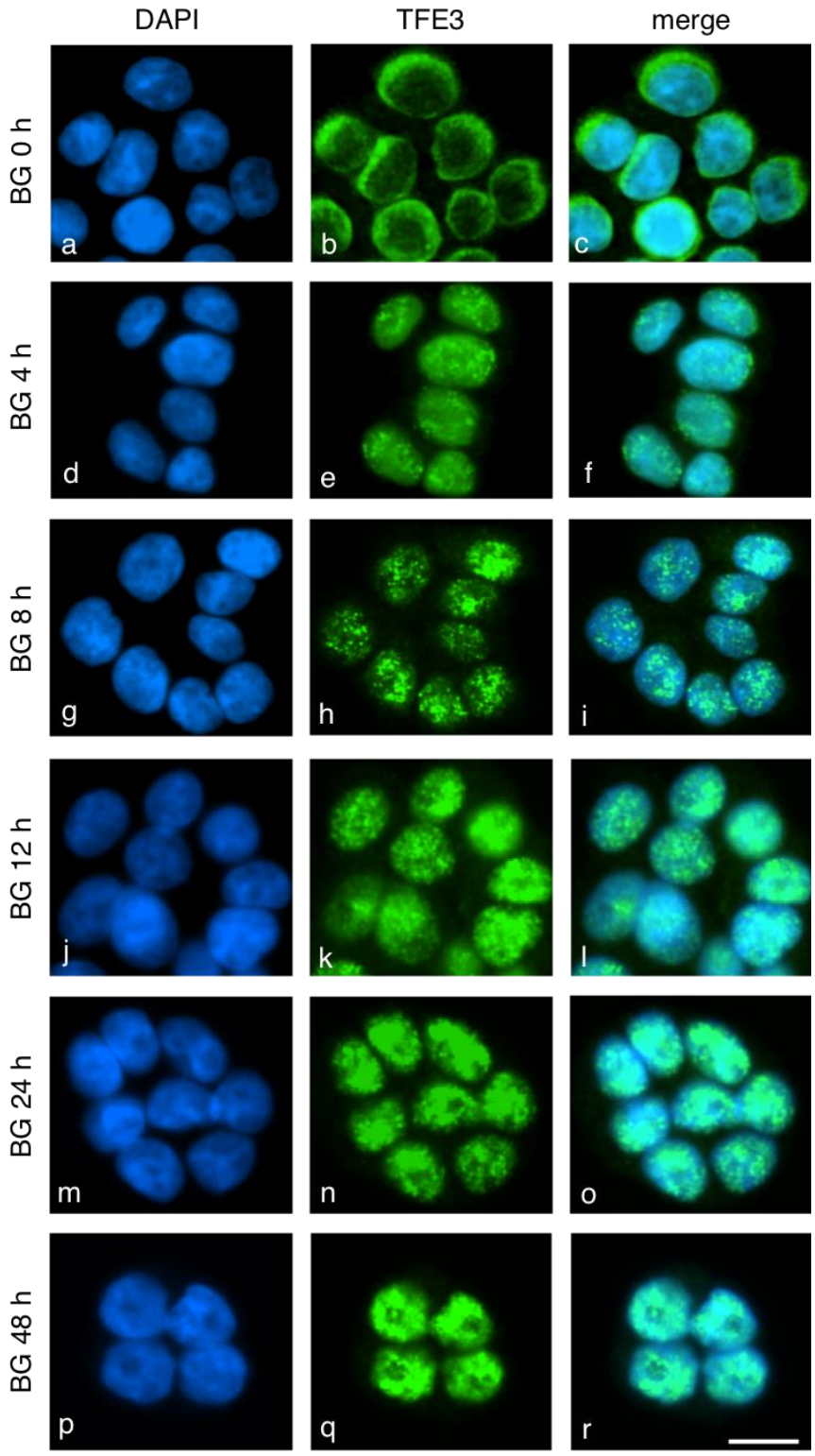


Fig. 15. Nuclear translocation of TFE3 upon BG treatment. HT29 cells treated with 10 mM of BG for the indicated times were subjected to immunocytochemistry using anti-TFE3 antiserum and DAPI for staining. Bars = 20 μM.

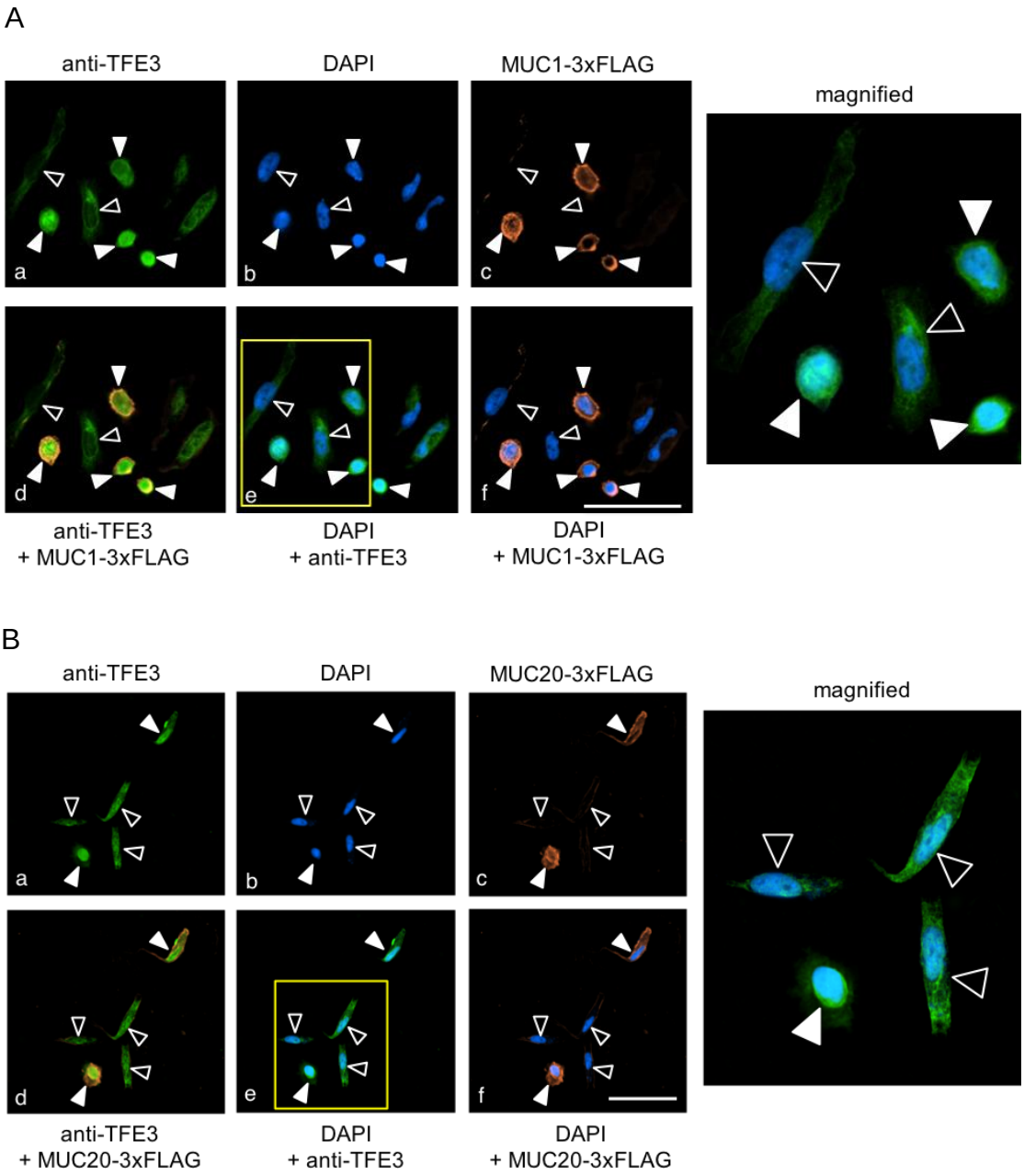


Fig. 16. Nuclear translocation of TFE3 upon MUC1 or MUC20 overexpression. (A) HeLa cells transfected with a 3×FLAG-MUC1 expression vector were stained with anti-FLAG (orange) and anti-TFE3 (green) antisera. Bars = 50 μm. Closed and open arrow heads indicate cells that overexpressed or did not overexpress 3×FLAG-MUC1, respectively. (B) HeLa cells transfected with a 3×FLAG-MUC20 expression vector were stained with anti-FLAG (orange) and anti-TFE3 (green) antisera. Bars = 50 μm. Closed and open arrow heads indicate cells that overexpressed and did not overexpress 3×FLAG-MUC20, respectively.

Third, we evaluated transcriptional induction from GASE upon mucin-type Golgi stress (Fig. 17). Vector control and 4xGASE mutant were used as a negative control for this experiment. As expected, overexpression of TFE3 increased transcription from GASE (lane 2, blue bar), but not from mutant GASE or vector control (lanes 1 and 3, blue bar). Surprisingly, BG-treated HT29 cells did not induce transcription from GASE, denoting that GASE cannot be activated by BG treatment alone. This suggests that mucin-type Golgi stress increases transcription, dephosphorylation, and nuclear translocation of TFE3 but it is not sufficient for transcriptional induction from GASE (see discussion). Although it can be noted that the P-value of lane 1 in Fig. 17A has one asterisk, it appears that luciferase activity was too low which leads to the fluctuation of the P-value

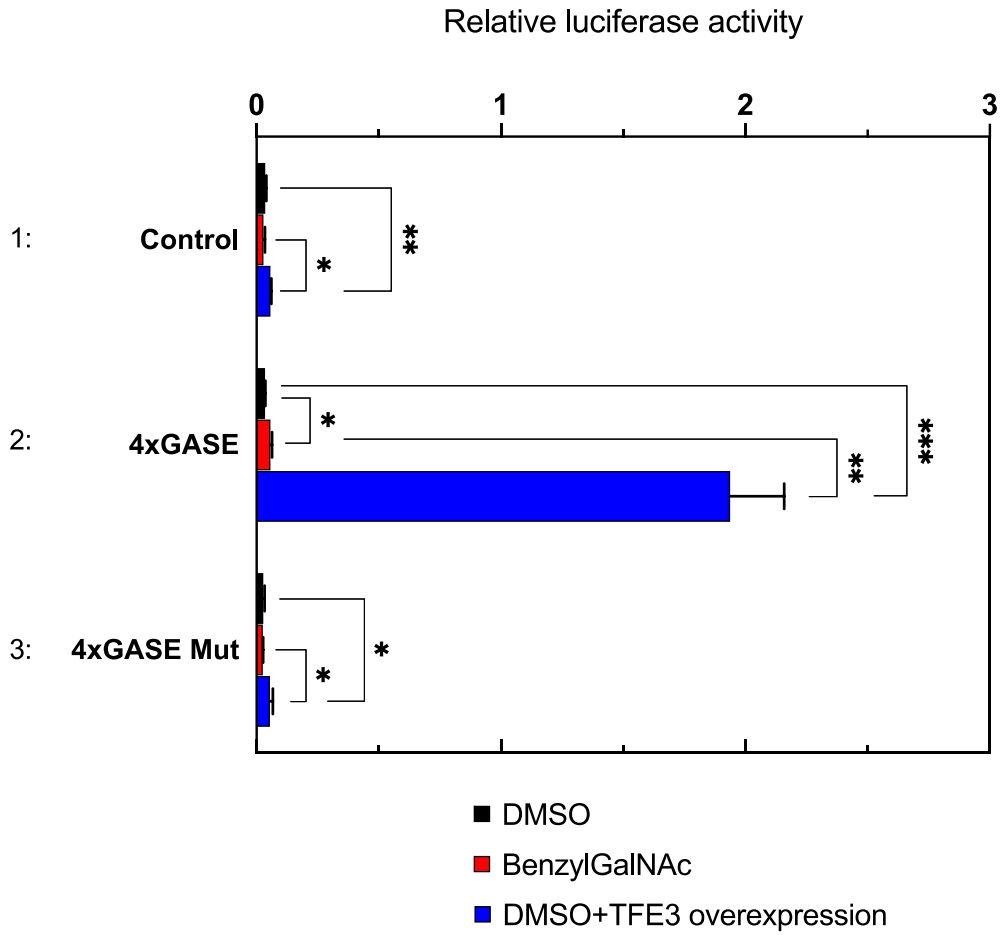


Fig. 17. Effects of BG treatment on GASE-mediated transcriptional induction. HT29 cells were transiently transfected with the indicated plasmids and subjected to luciferase assays. Reporter plasmid pGL3pro was used as the control, and is shown in lane 1. Experiments were repeated five times. ***, P < 0.001; **, P < 0.01; *, P < 0.05.

CHAPTER FOUR
DISCUSSION

The Golgi stress response is gradually recognized as one of the organelle autoregulation systems. However, the molecular mechanisms how the Golgi apparatus senses the insufficiency of its functional capacity (Golgi stress) to instigate reestablishment of its organelle function has been yet to be discovered. Currently, at least five mammalian Golgi stress response pathways have been reported: the TFE3, HSP47, CREB3, MAPK-ETS, and PGSE pathway. Among these pathways, Miyata *et al.*, 2013 identified the HSP47 pathway of the mammalian Golgi stress response, which is important for suppression of apoptosis induced by Golgi stress. Interestingly, the HSP47 pathway is activated by BenzylGalNAc, a chemical compound that can inhibit mucin glycosylation enzymes in the Golgi, which leads to insufficient mucin-type glycosylation. Mucin-type glycosylation of proteins is one of the important function of the Golgi apparatus, suggesting that inhibition of mucin-type glycosylation by BG may lead to accumulation of improperly modified mucin core proteins, and that mucin-secreting cells have to augment the expression of enzymes via an unidentified pathway of the Golgi stress response, that is, the mucin pathway.

In this study, we observed that upon insufficiency of mucin-type glycosylation (mucin-type Golgi stress), the expression levels of GALNT5, GALNT8 and GALNT18 were increased, thus, we named this novel pathway the mucin pathway. Strikingly, an unanticipated finding in the similar analysis was that incubation of cells with mucin-type Golgi stress induced the expression of TFE3 mRNA, a transcription factor regulating the TFE3 pathway of the Golgi stress response, implying the existence of crosstalk from the mucin pathway to the TFE3 pathway. Here, through analysis of the human TFE3 promoter and its mutants, we have identified a novel enhancer regulating transcriptional induction of the TFE3 gene upon mucin-type Golgi stress which we named MGSE, of which the critical sequence was ACTTCC(N9)TCCCCA. Furthermore, we found that TFE3 is dephosphorylated and translocates into the nucleus upon mucin-type Golgi stress, although transcription from GASE was not affected by mucin-type Golgi stress. From these observations, we concluded that the mucin pathway generates a crosstalk signal to the TFE3 pathway.

Further analysis of MGSE demonstrates that the activity of MGSE is dependent on the distance between the ACTTCC and TCCCCA motif which indicates that the distance that separates these two motifs is critical. It is possible that the transcription factors that bind to MGSE formed a heterodimer or two distinct transcription factors may synergistically bind to each portion. A similar situation is reported in the case of the ER stress response element (ERSE; consensus sequence CCAAT(N9)CCACG), an enhancer regulating the ATF6 pathway of the mammalian ER stress response (Yoshida et al., 1998). The transcription factor NF-Y binds to the CCAAT sequence, and another transcription factor, ATF6, requires both the CCACG sequence and NF-Y binding to the CCAAT sequence for its binding. Because the distance of 9 nt almost corresponds to one turn of a DNA double helix, transcription factors binding to MGSE seem to interact each other like ATF6 and NF-Y (Yoshida *et al.*, 2001).

We have been trying to identify an enhancer element that regulates transcriptional induction of GALNT5, GALNT8, and GALNT18 in response to mucin-type Golgi stress, and it is still unknown whether it shares any similarity or consensus with MGSE. Based on our survey of database, no MGSE-like sequences can be found in the human GALNT5, GALNT8, and GALNT18 promoters, suggesting that the enhancer element is unique from MGSE. As for an enhancer element regulating the transcriptional induction of HSP47 after BG treatment, we reasoned that it is distinct from MGSE and the enhancer for GALNTs, because (1) HSP47 promoter bears no obvious resemblance to MGSE sequence, and (2) the HSP47 pathway regulates a quite distinctive response compared to the mucin pathway, which is unrelated with glycosylation, rather, it suppresses apoptosis upon Golgi stress.

In comparison with potent activators of the TFE3 pathway such as monensin, BG seems to have distinct mode of action because: (1) BG upregulates expression of the TFE3 mRNA, whereas, monensin does not affect the expression of TFE3 mRNA (Fig. 9F) (Taniguchi *et al.*, 2015); and (2) monensin activates the TFE3 pathway, including nuclear translocation and dephosphorylation of TFE3 as well as transcriptional induction from GASE (Oku *et al.*, 2011), whereas BG, however, induces nuclear translocation and dephosphorylation of TFE3 only (Fig. 14 and Fig. 15) but unable to upregulate transcriptional induction from GASE (Fig. 17). Thus, BG does not stimulate a sensor for the TFE3 pathway and cannot trigger full activation of TFE3 pathway (including induction of GASE). In addition, there are two kinds of crosstalk signals from the mucin pathway to the TFE3 pathway, namely a signal enhancing

transcription of the TFE3 gene and a signal inducing dephosphorylation of TFE3 protein (Fig. 18). This raises the following three questions. First, why BG-treated cells did not activate transcription from GASE? We speculated that it is because cells have to avoid accidental activation of the TFE3 pathway. If the mucin pathway directly activated transcription from GASE, it could be induced without activation of the TFE3 pathway. Second, how do cells suppress ectopic transcriptional activation from GASE upon BG treatment? It is notable that crosstalk signals induced by BG treatment increases expression, dephosphorylation and nuclear translocation of TFE3, but it is not enough for transcriptional activation from GASE. We speculated that full activation of the TFE3 pathway may require additional signals, possibly additional post-translational modifications of TFE3 protein or transcription factors other than TFE3, which are induced or activated by the TFE3 pathway. Third, what is the biological significance of these crosstalk signals? We assumed that mucin-secreting cells require very high general Golgi capacity such as vesicular transport and N-glycosylation, because they produce high numbers of mucin proteins. Thus, cells enhance the TFE3 pathway using these crosstalk signals during differentiation of mucin-secreting cells such as goblet cells.

In the case of the ER stress response, crosstalk exists between the IRE1 and ATF6 pathways. pATF6(N), a transcription factor that regulates the ATF6 pathway (Yamamoto *et al.*, 2007), increases the transcription of XBP1 (Yoshida *et al.*, 2000), which is a key transcription factor regulating the IRE1 pathway (Yoshida *et al.*, 2003). Thus, the ATF6 pathway enhances the response of the IRE1 pathway. However, the ATF6 pathway only partially activates the IRE1 pathway, because ATF6 just increases the expression of XBP1 pre-mRNA, which has to be converted to mature mRNA by IRE1-mediated non-conventional mRNA splicing in order to produce an active transcription factor pXBP1(S) (Fig. 18) (Yoshida *et al.*, 2001).

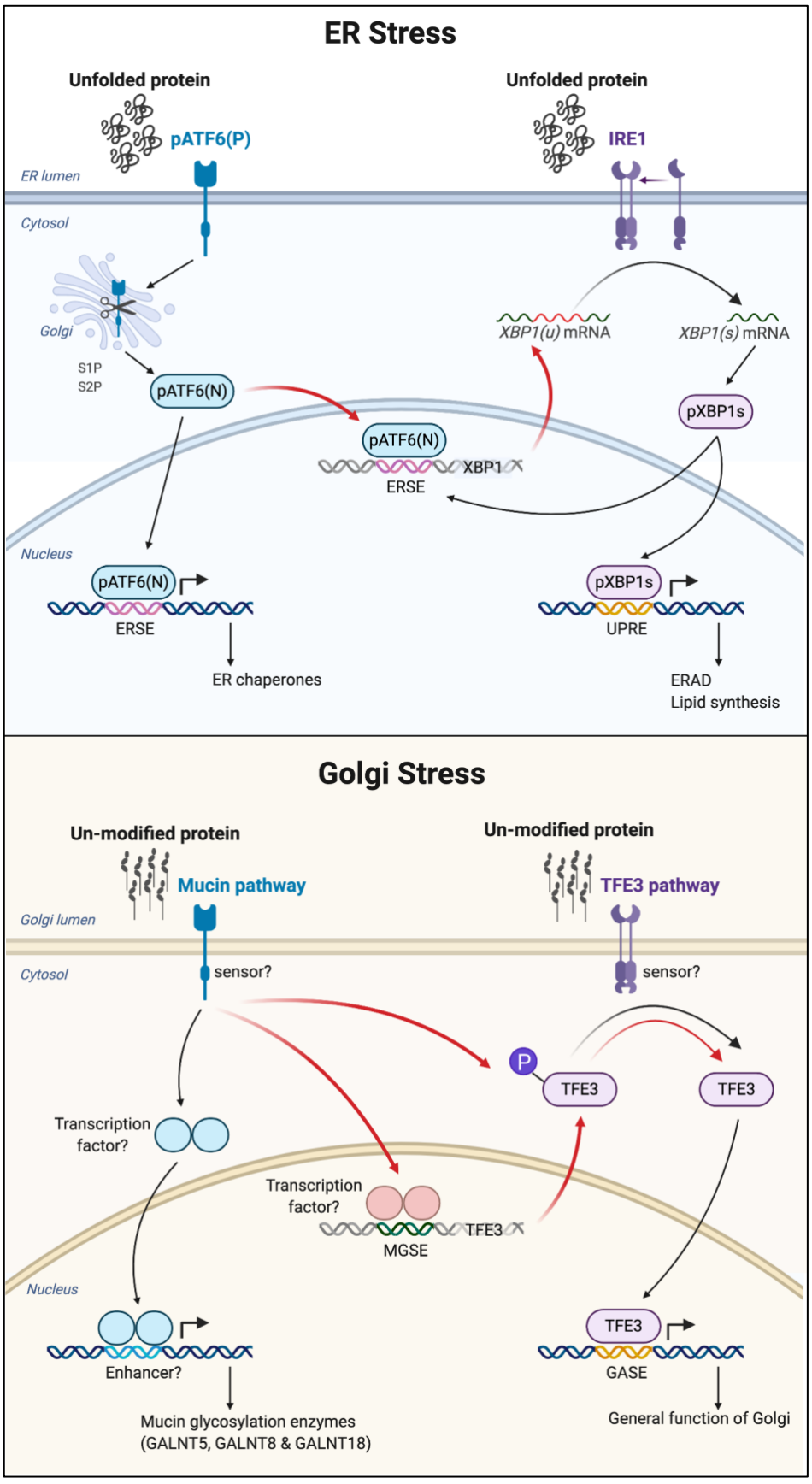


Fig. 18. Working hypothesis of crosstalk signaling from the mucin pathway to the TFE3 pathway. Crosstalk from the ATF6 pathway to the IRE1 pathway in the ER stress response pathway is also indicated.

Like the ER and Golgi stress responses, many studies are now progressing to clarify the mechanism that augments the capacity of each organelle in accordance with cellular demands, including the lysosomal stress response (Napolitano and Ballabio, 2016), the mitochondrial unfolded protein response (Shpilka and Haynes, 2018), and the peroxisome stress response, which are collectively called organelle autoregulation (Sasaki and Yoshida, 2015). Elucidation of the mechanisms of organelle autoregulation is one of the most fundamental issues in cell biology, because they are essential for the autonomy of eukaryotic cells. Insufficiency of functional capacity is sensed by specialized sensors localized on or in each organelle, which relay signals to transcription factors to augment their functions. Subsequently, transcription factors bind to unique enhancer elements to increase the expression of organelle-specific genes. In order to understand the perspective of organelle autoregulation, clarification of these regulators is crucial.

CHAPTER FIVE
CONCLUSIONS AND FUTURE DIRECTION

5.1 Conclusions

Identification of the mucin pathway of the mammalian Golgi stress response has diversified the current niche of organelle autoregulation, particularly in the Golgi stress response. Our current analyses have shown that the perturbation of mucin O-glycosylation or excessive number of mucin core proteins can lead to chaos and stress inside the Golgi apparatus. Thus, the Golgi activates the novel response pathway of the Golgi stress response (the mucin pathway) in order to maintain its homeostasis by inducing expression of glycosylation enzyme genes such as GALNT5, GALNT8, and GALNT18. Moreover, we identified a novel enhancer element MGSE and the unanticipated existence of crosstalk among mammalian Golgi stress response pathways, which will contribute to identifying the sensors and the transcription factors. Delineating the details of the mucin pathway could uncover more discovery towards understanding the mucus and facilitate the new development of therapeutic strategies for Golgi- or mucus-associated diseases.

5.2 Future Direction

Future works are required to identify a transcription factor(s) that enhances the TFE3 transcription from MGSE upon mucin-type Golgi stress. We will try to isolate the transcription factor by adopting yeast one-hybrid screening method. In brief, tandem repeats of the MGSE sequence will be ligated upstream of the yeast HIS3 gene. This plasmid will then be integrated into HIS3-deficient yeast cells (KMY1015) together with human pancreas cDNA library fused with the activation domain of yeast transcription factor GAL4, and transfected yeast cells were incubated on growth plates lacking histidine. Human cDNA will be recovered from yeast cells that can grow in the absence of histidine in order to identify the candidates of the transcription factors binding to MGSE. However, if two distinct gene products are required for binding to MGSE, Y1H screening cannot identify the transcription factors. In that case, CRISPR-

Cas9 screening will then be adopted, and the experiment conditions should be optimized for data collection and analysis.

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PUBLICATION

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MGSE regulates crosstalk from the mucin pathway to the TFE3 pathway of the Golgi stress response

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