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# Quality Control in the Early Secretory Pathway Plays Significant Roles in vivo

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## 1. Introduction

The endoplasmic reticulum (ER) provides a folding environment for newly synthesized secretory and membrane proteins (Ellgaard and Helenius, 2003). Secretory proteins are synthesized by ribosomes and translocated cotranslationally or posttranslationally to the ER. These newly synthesized proteins interact with ER molecular chaperones, such as immunoglobulin heavy chain-binding protein (BiP), calnexin, calreticulin, and protein disulfide isomerase, to become properly folded and assembled into a mature protein complex for transport along the secretory pathway. Aberrant protein folding, due to extracellular stimuli such as ischemia and oxidative stress, or genetic mutation leads to the accumulation of misfolded proteins in the ER, which in turn evokes the unfolded protein response (UPR) (Patil & Walter, 2001). The UPR reduces the amount of misfolded proteins (Ron & Walter, 2007) by inducing the production of ER chaperones that promote protein folding, reducing general protein synthesis, and enhancing the degradation of misfolded proteins via a ubiquitin-proteasome system, termed ER-associated degradation (ERAD) (Bonifacino & Weissman, 1998). The persistent accumulation of misfolded proteins beyond the capacity for ER quality control causes cellular dysfunction and cell death, leading to diverse human disorders, such as diabetes mellitus and neurodegenerative diseases (Zhao & Ackerman, 2006).

Misfolded proteins had been believed to remain in the ER, but yeast genetic analyses have indicated that the UPR involves the whole secretory pathway (Travers et al., 2000), and that some misfolded proteins require transport between the ER and the Golgi complex for ERAD (Vashist et al., 2001). In addition, certain misfolded proteins in mammalian cells have also been reported to exit the ER and recycle between the ER and post-ER compartments, associating with ER chaperones. The KDEL receptor mediates this retrieval, suggesting that the secretion of misfolded proteins with ER chaperones from the ER and their retrieval may contribute to ER quality control (Hammond & Helenius, 1994; Yamamoto et al., 2001).

We focus on the KDEL receptor-ER chaperone retrieval system in order to explore the physiological and pathological significance of ER quality control by using culture cells and mutant mouse models. The impairment of the retrieval system in our mutant mice caused several developmental and aging dysfunctions, which reveals that quality control in the early secretory pathway plays significant roles in vivo.

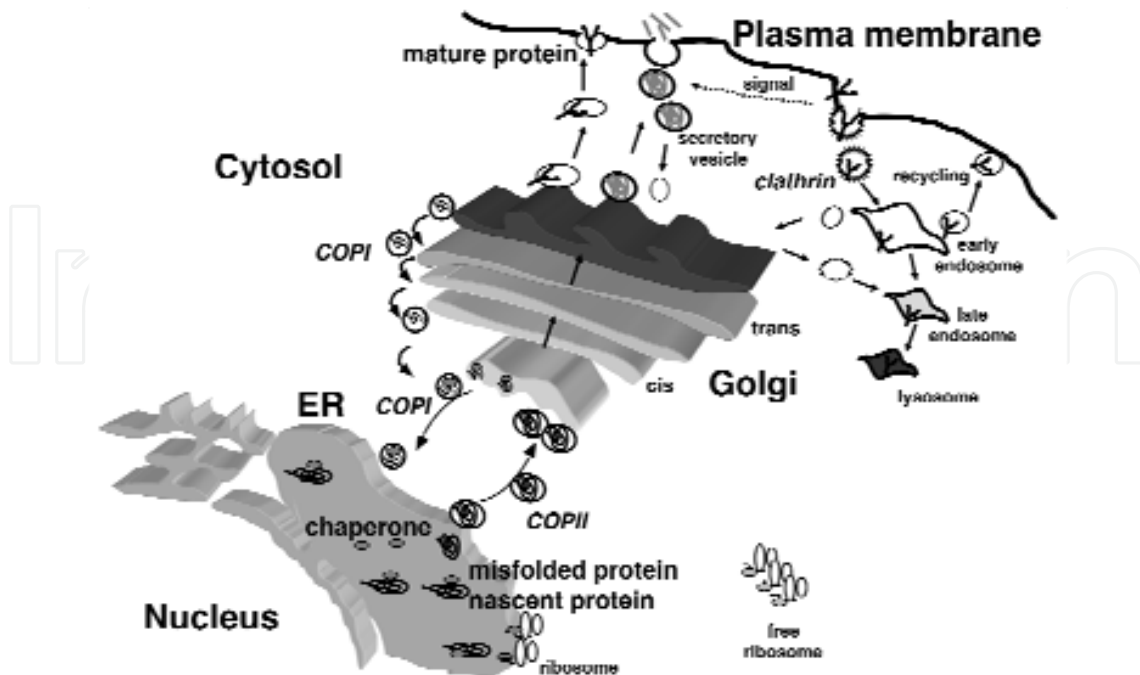


Fig. 1. Secretory pathways in the cell

### 1.1 Transport between the ER and the Golgi complex

Intracellular transport of proteins is mediated by membrane carriers that include coated vesicles and tubular structures. From the ER, proteins are packaged into the coat protein complex (COP) II coated vesicles to initiate their transport from the ER (Schekman and Orci, 1996). Subsequently, proteins are transferred to the intermediate compartment (IC) that then moves along microtubules to the Golgi apparatus (Bonfanti et al., 1998). Upon arrival to the Golgi complex, proteins are sorted to the peripheral compartments of the cell, such as endosomes and plasma membrane. Also, proteins can be retrieved to the ER by retrograde transport at either the IC or the Golgi complex by COPI coated vesicles (Letourneur et al., 1994).

Selective retrograde transport of proteins from post-ER compartments to the ER is achieved by multiple mechanisms. Transmembrane proteins that have a carboxyl terminal dilysine (KKXX) sequence, such as type I ER membrane proteins (Nilsson et al., 1989) and ERGIC53 (Itin et al., 1995), are transported in COPI vesicles, because the COPI coat recognizes the dilysine motif directly (Letourneur et al., 1994). However, soluble proteins that reside in the lumen of membrane compartments can also be retrieved to the ER. Ones such as BiP (or GRP78) have a carboxyl terminal Lys-Asp-Glu-Leu (KDEL) sequence (Munro & Pelham, 1987) that is recognized by the KDEL receptor in the post-ER compartments (Lewis & Pelham, 1992), and are then sorted into COPI vesicles for retrograde transport (Orci et al., 1997).

Besides its retrieval function, the KDEL receptor has been suggested to play a role in regulating COPI transport (Aoe et al., 1997; Aoe et al., 1998). ADP-Ribosylation Factor 1 (ARF1), a Ras-like small GTPase, regulates the formation of COPI vesicles (Rothman & Wieland, 1996). Like all small GTPases, the activation of ARF1 requires a guanine nucleotide

exchange factor, and its deactivation requires a GTPase-activating protein (GAP). GAP acts by promoting the hydrolysis of GTP on ARF1 to GDP (Cukierman et al., 1995). This action has been demonstrated to be required for the proper formation of COPI vesicles (Hsu et al., 2009). Significantly, ligand-binding on the luminal side of the KDEL receptor also induces its interaction with ARFGAP1 on the cytoplasmic side of the receptor. As a result, ARFGAP1 is recruited from the cytosol to the membranes to activate GAP activity on ARF1 (Aoe et al., 1999). Thus, these findings suggest that the KDEL receptor does not merely function as a passive cargo protein of COPI transport, but is capable of modulating this transport pathway.

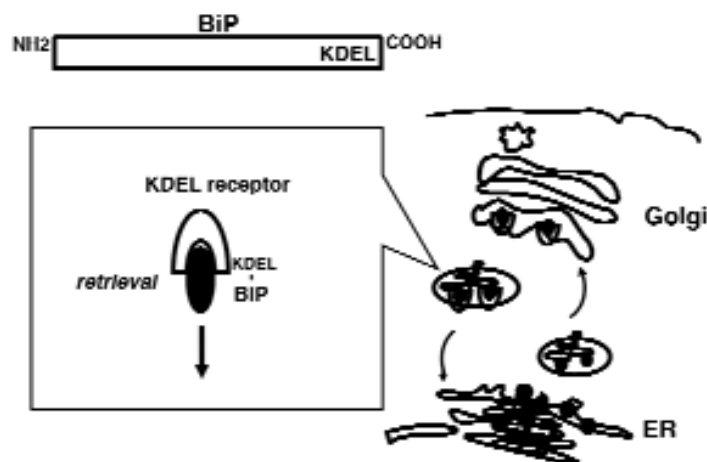


Fig. 2. ER chaperones with the KDEL sequence are retrieved by the KDEL receptor

### 1.2 ER chaperones and the unfolded protein response

BiP, one of the most abundant ER chaperones, plays a central role in ER function, assisting in protein translocation, folding, degradation and regulation of the UPR (Hendershot, 2004). ER chaperones are localized to the ER by two mechanisms—retention and retrieval (Sonnichsen et al., 1994). BiP is retained in the ER through interaction with other ER proteins and the ER matrix. When misfolded proteins accumulate in the ER, BiP dissociates from some ER membrane proteins, such as inositol-requiring kinase-1 (IRE1), PKR-like ER-associated kinase (PERK) and activating transcription factor 6 (ATF6). BiP dissociation activates these kinases and transcription factors and initiates the UPR (Bertolotti et al., 2000), which leads to increased expression of X-box-binding protein-1 (XBP-1) and ATF4 (Schroder & Kaufman, 2005). When BiP is secreted from the ER along with misfolded proteins (Hammond & Helenius, 1994; Yamamoto et al., 2001), the KDEL sequence of BiP is recognized by the KDEL receptor, thereby facilitating the retrieval of BiP from post-ER compartments to the ER (Lewis & Pelham, 1990; Munro and Pelham, 1987). Yeast BiP (Kar2) is essential for survival; when the retrieval sequence (in yeast: His-Asp-Glu-Leu, HDEL) is deleted, a fraction of Kar2 is secreted from the ER. However, the UPR is activated and this maintains a minimal level of Kar2 in the ER (Beh and Rose, 1995). Thus, the failure of retrieval by the KDEL receptor causes the UPR.

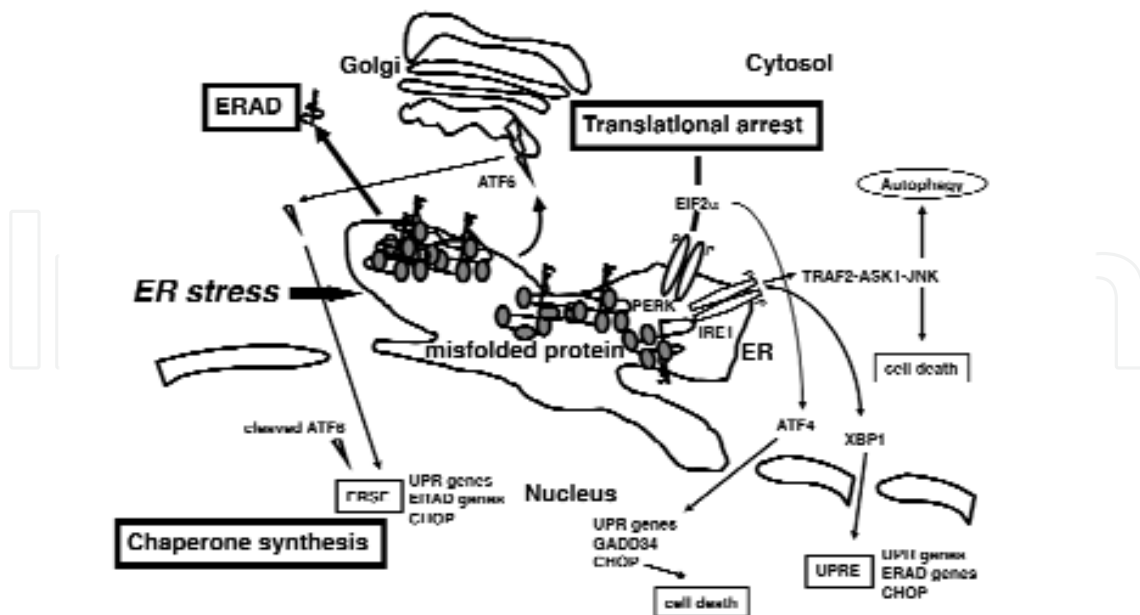


Fig. 3. ER stress causes the UPR, leading to chaperone synthesis, translational arrest and ERAD

## 2. The role for retrieval of misfolded proteins by the KDEL receptor in ER quality control examined by using culture cells

We examined how the KDEL receptor-ER chaperone retrieval system participates in ER quality control by studying the unassembled T cell antigen receptor (TCR)  $\alpha$  chain as a model system (Yamamoto et al., 2001).

### 2.1 TCR $\alpha$ cycles in the early secretory system

TCR consists of at least six polypeptides (TCR $\alpha$ ,  $\beta$ , CD3 $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ ). Proper assembly is required for TCR to be expressed on the cell surface (Klausner et al., 1990). A heterologous expression system has been used extensively to study the fate of unassembled TCR $\alpha$  chain by transfecting TCR $\alpha$  into COS cells that normally do not express the TCR. Unassembled TCR $\alpha$  has been demonstrated to be retained in the ER (Bonifacino et al., 1989) and degraded by ERAD in lymphocytes, as well as other cell types when it is expressed as a single subunit (Huppa & Ploegh, 1997).

However, because an increasing number of proteins have been appreciated to maintain their distribution in the ER by being transported out and then cycling back, we tested whether TCR $\alpha$  might also exhibit such a behavior. Upon treatment with bafilomycin A1, a vacuolar H<sup>+</sup>-ATPase inhibitor that has been shown to impose a relative block on the retrograde arm of bidirectional transport between the ER and the Golgi complex (Palokangas et al., 1998), confocal microscopy revealed significantly increased colocalization of TCR $\alpha$  and ERGIC53. The colocalization induced by bafilomycin also involved the redistribution of ERGIC53 to a pattern that was more compact and juxtannuclear. That is, when retrograde transport was blocked, ERGIC53 could be induced to accumulate at the Golgi complex (Palokangas et al., 1998).

Because of these known effects on the itinerary of proteins cycling in the early secretory compartments, we sought to examine the distribution of TCR $\alpha$  in more detail by subcellular fractionation. In the control condition, when no perturbation was added, most of TCR $\alpha$  was in the ER fractions as a p38 form, as judged by co-fractionation with calnexin. In this control setting, we could also detect a larger form (p43) in post-ER fractions, as judged by co-fractionation with the IC marker. As previous characterization of TCR $\alpha$ , p38 is an immature glycoprotein that has not received Golgi-specific modification, while p43 represents a mature glycoprotein that has received Golgi-specific modification (Samelson, 1985). Consistent with the possibility that TCR $\alpha$  cycles out of the ER, when cells were treated with bafilomycin, TCR $\alpha$  accumulated markedly in post-ER fractions as the p43 form. To ascertain that the p43 form of TCR $\alpha$  indeed represented those outside the ER, we assessed whether it had Golgi-specific glycosylation. Immunoblotting for TCR $\alpha$  from the different fractions that were either treated with endoglycosidase H (endo H) or subjected to mock-treatment, we found that p38 form was endo H sensitive, while the p43 form was endo H resistant. These results, taken together with the above morphologic findings, suggest that a significant fraction of TCR $\alpha$  was cycling between the ER and the Golgi complex.

## 2.2 Retrieval of TCR $\alpha$ by the KDEL receptor

A dilysine (KKXX) motif on the cytoplasmic portion of transmembrane proteins mediates their retrograde transport to the ER, because COPI binds directly to this motif (Letourneur et al., 1994). While TCR $\alpha$  is a transmembrane protein, it contains no such motif in its cytoplasmic domain. However, another possibility is suggested by studies on the ER chaperone, BiP. BiP has been shown to leak out to the post-ER compartments, and then retrieved to the ER, because it contains a carboxy terminal KDEL motif that is recognized by the KDEL receptor (Munro & Pelham, 1987). Thus, one possibility is that the KDEL receptor can retrieve TCR $\alpha$ , if TCR $\alpha$  were bound to a KDEL ligand, such as BiP. Consistent with this possibility, co-precipitation studies revealed an association between TCR $\alpha$  and BiP. This association was specific, because another ER chaperone, GRP94, did not co-precipitate with TCR $\alpha$ .

To gain insight into the importance of the retrieval function of the KDEL receptor, we examined whether TCR $\alpha$  could be detected on the cell surface upon disruption of ligand-binding by the KDEL receptor. Whereas such a surface pool was undetectable when retrieval by the KDEL receptor was intact, a significant fraction was detected when retrieval was disrupted by the overexpression of lysozyme-KDEL, a chimeric KDEL ligand, that was created by appending the KDEL sequence to lysozyme.

We had shown previously that activation of the KDEL receptor through ligand-binding on its luminal side induced the KDEL receptor to interact with the ARFGAP1 on the cytoplasmic side (Aoe et al., 1998). Thus, one possibility is that the complex of TCR $\alpha$  with BiP represented a ligand that would also activate the KDEL receptor to interact with GAP. Upon overexpression of TCR $\alpha$ , we detected an increased interaction between the KDEL receptor and ARFGAP1. Thus, this finding suggested that, most likely, TCR $\alpha$  was being retrieved by the KDEL receptor, because it was complexed with BiP, a known ligand for the receptor.

Taken together, these observations suggested that ligand binding by the KDEL receptor would regulate the degree of COPI-mediated retrograde transport. During stress that increases the level of misfolded proteins in the ER, one possibility is that more of these

misfolded proteins would leak from the ER. However, because of the mechanistic link between ligand-binding by the KDEL receptor and its interaction with ARFGAP1, an important function of the KDEL receptor appears to be in integrating increased leakage that reaches the Golgi complex to increasing COPI-mediated retrograde transport from this organelle. In this manner, any increased leakage out of the ER would not result in increased leakage out of the early secretory system. This study suggested an important role for the KDEL receptor in a post-ER retrieval mechanism that contributes to ER quality control (Yamamoto et al., 2001).

### **3. The role for retrieval of misfolded proteins by the KDEL receptor in ER stress response examined by using culture cells**

The mammalian ER stress response is a complicated process that is coordinately induced by ER transmembrane kinases such as ATF6, IRE1 and PERK. Further complexity comes from the fact that the activation of these kinases not only enhances the expression of ER chaperones for cytoprotection but also causes apoptosis. The underlying molecular mechanism of the transition between these two opposite outcomes during the ER stress response is uncertain.

We examined how the KDEL receptor-ER chaperone retrieval system participates in ER stress response by using an expression of a mutant KDEL receptor that lacks the ability for ligand recognition. The impairment of retrieval by the KDEL receptor led to a mis-sorting of BiP, an ER chaperone that had a retrieval signal from the early secretory pathway, which induced an increase in susceptibility to ER stress in HeLa cells (Yamamoto et al., 2003).

#### **3.1 Ligand recognition by the KDEL receptor is saturable**

Transient transfection of the KDEL receptor results in various expression levels in HeLa cells. Overexpression of the KDEL receptor has been shown to enhance transport from the Golgi complex to the ER, which results in the redistribution of the whole Golgi complex to the ER (Hsu et al., 1992); however, moderate expression of the KDEL receptor does not disturb the Golgi structure and only those cells with moderate expression become stable transfectants (Aoe et al., 1998). To examine the role of the KDEL receptor in the ER stress response, we made HeLa cells that stably expressed either the wild-type or a mutant human KDEL receptor. This binding defective mutant that had a single amino acid replacement (R169N) was localized to the Golgi complex as the wild-type, but did not respond to the KDEL sequence effectively as described previously (Townsend et al., 1993). The transient expression of the lysozyme-KDEL in these stable cells induced the transport of the wild-type KDEL receptor, but not that of the mutant, to the ER. The lysozyme-KDEL was localized in the ER even in the mutant KDEL receptor cells, which indicates that minimal retrieval was maintained in these cells by the endogenous KDEL receptor.

The expression of ER chaperones is induced extensively upon ER stress, which may saturate KDEL receptor-mediated retrieval since the expression of the KDEL receptor is not inducible in mammals. We examined the effect of ER stress on the distribution of BiP, an endogenous ligand of the KDEL receptor, in the secretory pathway, using sucrose gradient analysis in cells stably expressing the wild-type or the mutant KDEL receptor. While most endogenous BiP was found in the ER in the resting state, a significant amount was detected in the post-ER fractions of these cells when the cells were treated with tunicamycin, which prevented

protein glycosylation in the ER and induced the UPR. Under these circumstances, we detected more BiP being secreted to the medium in the mutant KDEL receptor cells than in the HeLa cells and the wild-type KDEL receptor cells in spite of an equivalent expression level of BiP within the cells. These results suggested that ER chaperones associating with misfolded proteins might be secreted from the ER and retrieved by the KDEL receptor. When retrieval is limited, they may escape from the early secretory pathway, especially under stressed conditions.

### **3.2 The loss of BiP from the early secretory pathway causes persistent UPR**

The accumulation of misfolded proteins in the ER leads to the recruitment of BiP from ER transmembrane kinases such as ATF6 and IRE1, which results in the activation of these kinases and the synthesis of ER chaperones including BiP. The loss of BiP in the early secretory pathway may enhance this process. We examined the induction of BiP gene transcription with Northern blot analysis. While the BiP mRNA in all cells increased upon tunicamycin treatment, it was prominent and persistent in the mutant KDEL receptor cells. We also observed an increased phosphorylation of IRE1 upon tunicamycin treatment, even at the basal level, in the mutant KDEL receptor cells. These results indicated that retrieval by the KDEL receptor kept BiP in the early secretory pathway efficiently, and that the impairment of retrieval caused a loss of BiP there that induced intense UPR.

We assessed whether retrieval by the KDEL receptor might contribute to cell survival in the UPR. HeLa cells and the wild-type or the mutant KDEL receptor cells were treated with tunicamycin and DTT. Significantly, the mutant KDEL receptor cells were sensitive to ER stress, while cells expressing the wild-type tolerated the stress. By TUNEL assay, we detected more apoptotic features in the mutant KDEL receptor cells undergoing tunicamycin treatment. Hoechst 33258 staining revealed prominent condensed chromatin and the fragmentation of nuclei in the mutant KDEL receptor cells with tunicamycin. These features were consistent with programmed cell death by ER stress, suggesting that the retrieval of BiP and misfolded proteins from post-ER compartments by the KDEL receptor played a significant role in the ER stress response. When retrieval was limited, the susceptibility to ER stress might be increased (Yamamoto et al., 2003).

## **4. The effect of the impairment of the KDEL receptor-ER chaperone system *in vivo***

The KDEL receptor-ER chaperone system had been extensively studied in yeast and mammalian cells; however, its function and the resultant outcomes of its dysfunction in animals or humans *in vivo* were totally unknown. The impairment of the KDEL receptor was expected to perturb ER quality control, which might possibly cause diseases associated with ER stress (Hamada et al., 2004).

### **4.1 Generation of transgenic mice expressing the mutant KDEL receptor**

We took advantage of previous studies on the KDEL receptor and made stable cell lines expressing a transport mutant human KDEL receptor (Townsend et al., 1993). The mutant KDEL receptor (D193N) recognizes the KDEL proteins; however, the receptor is not transported to the ER upon ligand recognition but rather stays in post-ER compartments (Townsend et al., 1993). We found that the mutant KDEL receptor disturbed the circulation of misfolded proteins between the ER and the Golgi complex, resulting in the accumulation



of misfolded proteins in the ER. As a result, these cells became sensitive to ER stress. This finding prompted us to make transgenic (TG) mice expressing a mutant KDEL receptor possibly sensitive to ER stress. In order to investigate the function of the KDEL receptor *in vivo*, we created TG mice expressing the human wild-type or the transport mutant KDEL receptor, using CAG promoter. We established two lines of the wild-type and two lines of the mutant KDEL receptor TG mice. Northern blot revealed that the expression level of the mutant KDEL receptor was as high as that of the endogenous one. Western blot confirmed that the mutant KDEL receptor was expressed ubiquitously (Hamada et al., 2004).

#### **4.2 Dilated cardiomyopathy (DCM) caused by aberrant ER quality control in the mutant KDEL receptor transgenic mice**

These TG mice seemed to grow up normally until early adulthood. In the course of the study, we found that the transport mutant KDEL receptor TG mice of both lines died sporadically after the age of more than fourteen weeks. They appeared dyspneic, lethargic and motionless. These mice developed peripheral edema, ascites and cardiomegaly, and seemed to die due to heart failure. We observed marked four-chamber dilation without wall thickening. The average heart to body weight ratio of the mutant KDEL receptor TG mice was significantly higher than that of the wild-type KDEL receptor TG mice and the parental C57BL/6 mice. Histological examination revealed that the cardiomyocytes of the mutant TG mice were of varying sizes but were significantly enlarged on the average. There was also an expanded interstitial fibrosis and vacuolization. On the other hand, the histology of the wild-type TG mice appeared normal. Using TUNEL staining, we detected significantly more apoptotic cells in the hearts of the mutant KDEL receptor TG mice than in those of the control C57BL/6 mice and the wild-type TG mice. We found no significant difference in systemic arterial blood pressure between the mutant KDEL receptor TG mice and the C57BL/6 mice, suggesting that the cardiomegaly in the mutant mice was not a secondary change due to systemic hypertension. Taken together, these results suggested that the mutant KDEL receptor TG mice developed primary DCM.

#### **4.3 Ultrastructural analyses revealed the accumulation of protein aggregates in the expanded sarcoplasmic reticulum of the mutant cardiomyocytes**

In order to gain insight into the mechanism responsible for DCM in the mutant KDEL receptor TG mice, we examined the ultrastructure of the mutant cardiomyocytes. The contractile apparatus appeared intact, i.e. the arrangement of myofibril structures and the banding of myofilaments was little different in the myocardia of control C57BL/6 mice and mutant TG mice. The arrangement of mitochondria was also intact in the TG mice. On the other hand, the proliferation of sarcoplasmic reticulum (SR) was prominent around the transverse (T) tubule area, and the T tubule structure was narrowed significantly in both mutant TG mice with and without apparent cardiomegaly in comparison to the T tubules and surrounding the SR in the control. Furthermore, an aggregation of degenerated membrane structures was found among the proliferated SRs, and lamellated or further fused membrane structures were found in the T tubule area in the mutant mice. We observed electron dense materials, possibly protein aggregates in the expanded SR. These aggregates were associated with polyribosomes, suggesting that they were part of the ER network. On the other hand, the ultrastructure of the cardiomyocytes from the wild-type KDEL receptor TG mice appeared normal.

These morphological changes found in the cardiomyocytes of the mutant KDEL receptor TG mice suggested that the mutant KDEL receptor might have impaired ER quality control, which led to the accumulation of misfolded proteins in the SR.

#### **4.4 Hearts in the mutant KDEL receptor transgenic mice might have suffered from ER stress**

We took neonatal cardiomyocytes as a primary cell culture. When treated with tunicamycin, neonatal cardiomyocytes from the control mice showed a diffuse expression of BiP, apparently in the ER distribution. To the contrary, we observed cell shrinkage with uneven distribution of BiP in the cardiomyocytes of the mutant KDEL receptor TG mice, suggesting that those cardiomyocytes were sensitive to ER stress. An accumulation of misfolded proteins in the ER leads to protein degradation in a ubiquitin-proteasome system. Western blot revealed a marked accumulation of ubiquitinated protein aggregates in the hearts of the mutant KDEL receptor TG mice, not the wild-type KDEL receptor TG mice, suggesting that misfolded proteins might have saturated the ubiquitin-proteasome system during a circumstance in which the mutant KDEL receptor might have impaired the capacity for ER quality control. We found slightly increased expressions of BiP and significant accumulations of CHOP in the adult mutant hearts. Since CHOP is induced by ER stress and has been acknowledged to cause apoptosis during the UPR (Zinszner et al., 1998), this might account for the enhanced apoptosis in the mutant hearts. These results suggest that the mutant hearts might have suffered from ER stress, which might have contributed to the pathogenesis of the cardiomyopathy found in the mutant mice. Pressure load might contribute to the ER stress on the heart.

DCM is characterized by an increased ventricular chamber size and a reduced contractility of the heart typically accompanied by loss of cardiomyocytes (Narula et al., 1996). The mutant KDEL receptor TG mice demonstrated cardiac dilation with congestive heart failure, interstitial fibrosis, myocyte heterogeneity, vacuolization and apoptosis, which closely resembles the pathologic and clinical features of human DCM. Clinically and experimentally, DCM is caused by a variety of factors (Ross, 2002). ER stress brings on human disorders such as neurodegenerative diseases (Kopito & Ron, 2000); however, it was uncertain whether it also caused cardiac diseases.

Cardiomyocytes are exposed to mechanical stress throughout their lives. The mutant KDEL receptor transgene is driven by the CAG promoter and is supposed to express ubiquitously. The expression level is moderate, as high as that of the endogenous one. Although all tissues of the mutant KDEL receptor TG mice might be sensitive to ER stress, one of the most important factors that affect whether ER stress may cause a disease is the regenerative ability of the concerned tissue. Neurodegenerative diseases and diabetes mellitus are usually regarded as typical ER stress-associated diseases or conformational diseases (Kopito & Ron, 2000) because a neuron and a  $\beta$  cell live as long as the lifespan, and are therefore susceptible to the accumulation of misfolded proteins. In this regard, cardiomyocytes have the same lifespan, and they are exposed to harsh conditions, such as mechanical and oxidative stress. This study suggested that a cardiomyocyte might also be a possible target for ER stress (Hamada et al., 2004).

### **5. BiP, an ER chaperone, plays important roles in mammalian development**

BiP plays a central role in ER function. Yeast BiP (Kar2) is essential for survival; when the retrieval sequence (in yeast: His-Asp-Glu-Leu, HDEL) is deleted, a fraction of Kar2 is

secreted from the ER. However, the UPR is activated and this maintains a minimal level of Kar2 in the ER (Beh & Rose, 1995). Thus, the retrieval of BiP is not essential for a single cell. Complete depletion of BiP could be lethal for early embryonic cells in mice (Luo et al., 2006). We therefore produced knock-in mice expressing a mutant BiP in which the retrieval sequence was deleted by homologous recombination. These mice were used to elucidate any processes sensitive to ER stress during development and in adulthood (Mimura et al., 2007).

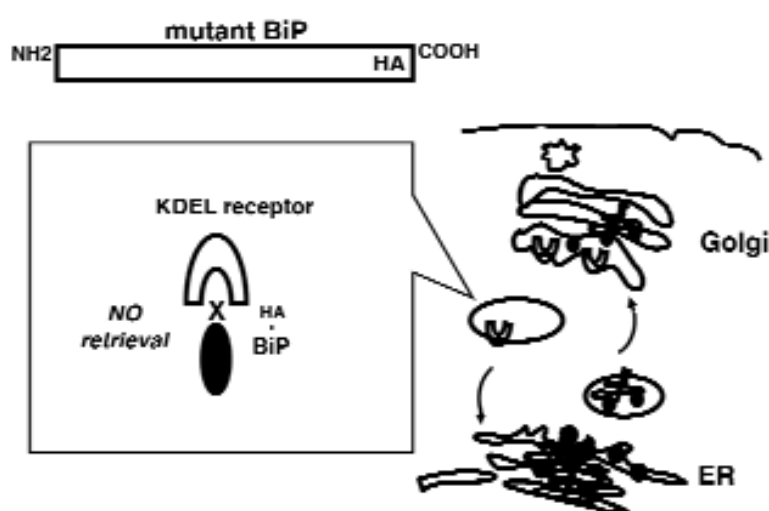


Fig. 4. The mutant BiP is defective in retrieval, leading to an escape of misfolded proteins from ER quality control

### 5.1 Constitutively active UPR compensates for loss of ER BiP in cultured mammalian cells

The mutant BiP contained a carboxyl-terminal HA tag in stead of a KDEL sequence. Mouse embryonic fibroblasts (MEFs) derived from homozygous BiP mutant embryos expressed the mutant BiP instead of wild-type BiP, and grew as well as wild-type MEFs. Mutant BiP localized to the ER, and its expression was enhanced by tunicamycin. These results were consistent with the expression for wild-type BiP and other ER chaperones containing a KDEL sequence. However, in metabolic labeling experiments, a significant fraction of mutant BiP was found in the medium, reflecting deletion of the KDEL sequence and impaired retrieval of mutant BiP. A fraction of wild-type BiP was also secreted into the medium during ER stress with tunicamycin treatment, indicating that retrieval by the KDEL receptor is saturable.

Tunicamycin sensitivity and expression of mutant BiP was also confirmed by Western blot. Both mutant BiP and wild-type BiP were recognized by an antibody against the amino terminus of BiP. However, an anti-KDEL antibody only recognized the wild-type BiP and GRP94, an ER chaperone with the KDEL sequence. Basal expression of XBP1, ATF4, phospho-PERK, GRP94 and another ER chaperone, calreticulin, was enhanced in homozygous mutant BiP MEFs. Basal expression of mutant BiP mRNA was also enhanced

in the mutant MEFs, indicating that the UPR was constitutively active. Thus, as seen previously in yeast (Beh & Rose, 1995), constitutive UPR activation maintained a minimal level of mutant BiP in the ER of mammalian cells, thus compensating for deletion of the KDEL sequence.

### 5.2 Mutant BiP embryos die shortly after birth

BiP was ubiquitously expressed in both mutant and wild-type embryos. In all tissues examined, GRP94 expression was greater in homozygous BiP mutant embryos than in wild-type embryos, suggesting that homozygous BiP mutant mice might suffer from global ER stress. Homozygous mutant BiP embryos weighed less than wild-types and heterozygotes at embryonic day (E) 18.5. Homozygous BiP mutant mice were born at the expected Mendelian ratio of 1:2:1. Neonatal BiP mutants moved well and responded to painful stimuli, but they appeared pale and cyanotic. They also cried less and displayed shallow breathing. The neonatal homozygous mutants generally died within several hours of birth; thus, we suspected that the observed lethality might reflect respiratory problems. When delivered by Caesarian section at E18.5 and sacrificed prior to breathing, gross morphology of the lungs and airways from BiP mutant mice was indistinguishable from wild-type. Wild-type and homozygous BiP mutant embryonic alveoli had an equivalent distribution of alveolar type II cells expressing the surfactant protein, SP-C. However, histological examination of lungs isolated from neonatal BiP mutants several hours after birth revealed atelectasis with poor inflation of peripheral airways. Hemorrhage and cell debris were also observed in the mutant alveolar space. Alveolar epithelia in BiP mutant mice were enlarged, whereas, as expected, those in wild-type neonates were distended. These observations indicated that homozygous neonatal BiP mutants developed atelectasis and respiratory failure after birth (Mimura et al., 2007).

### 5.3 Respiratory distress syndrome of newborns

Respiratory distress syndrome of newborns, also called hyaline membrane disease, causes high mortality and often accompanies preterm delivery or low birth weight with reduced expression of pulmonary surfactant. Surfactant therapy combined with mechanical ventilation and other intensive care measures has reduced the mortality rate of this syndrome to below 10% (Hallman, 2004). Surfactant proteins are required for proper lung development and function.

To examine whether a deficiency of pulmonary surfactant contributes to respiratory failure in homozygous BiP mutant mice, perfluorocarbon, a substitute for pulmonary surfactant, was administered into the oropharynx. Perfluorocarbon with oxygen treatment improved the activity of neonatal BiP mutants, turned their skin color from pale to pink, and improved lung inflation. The expression of surfactant proteins in neonatal lung was examined by Western blot. Expression of SP-A and, more prominently, proSP-C, was reduced in mutant lungs compared with wild-type, but there was no significant difference in proSP-B and SP-D expression. RT-PCR analysis revealed that the marked reduction of proSP-C in neonatal mutant lung was not due to reduced transcription. Importantly, after birth, the expression of proSP-C was enhanced only in wild-type neonates, suggesting that proSP-C might be degraded post-translationally in neonatal type II cells from BiP mutants.

Mature SP-B and SP-C are transported to the lamellar body where they bind phospholipids and are then secreted into the alveolar space via regulated exocytosis, whereas SP-A and SP-

D are secreted independently of the lamellar body. The subcellular localization of SP-A and SP-C was evaluated by confocal laser microscopy. In wild-type neonatal alveolar type II cells, SP-A and SP-C (proSP-C) colocalized with BiP, and other KDEL sequence-containing ER chaperones, in the ER. SP-A accumulated in the alveolar lining area of BiP mutant mice and costained with mutant BiP. By contrast, SP-C remained in the ER, and its expression was markedly reduced in type II cells of neonatal BiP mutants. Together, these data suggested that mutant BiP impaired the secretion of pulmonary surfactant, especially secretion through the lamellar body.

Embryonic type II cells store glycogen in the cytoplasm, and this glycogen is consumed as the synthesis of pulmonary surfactant expands after birth. Type II cells in neonatal BiP mutants contained vacuole structures. Periodic acid Schiff (PAS) staining revealed cytoplasmic polysaccharides in these cells, even after birth. Ultrastructural analysis of type II cells from neonates confirmed that cytoplasmic glycogen was indeed still present in mutant, but not in wild-type, cells. More importantly, the structure of the lamellar body was abnormal in embryonic and neonatal mutant type II cells. The lamellar body in wild-type neonates had wavy, dense laminations with clefting, whereas in BiP mutant neonates the lamellar body had loosely formed lamellar structures or was almost empty. These results indicated that the biosynthesis and secretion of pulmonary surfactant was impaired in BiP mutant type II cells (Mimura et al., 2007).

#### **5.4 Aberrant quality control in the endoplasmic reticulum causes neonatal respiratory failure in mice expressing mutant BiP**

SP-C is a small, highly hydrophobic protein processed from proSP-C during its transport through the ER and the Golgi to the multivesicular body. Mature SP-C is transported further to the lamellar body where it binds phospholipids before secretion into alveolar space via regulated exocytosis. Lamellar body formation is defective in alveolar type II cells of neonatal BiP mutants. Therefore, SP-C may be degraded by endosomal/lysosomal degradation and/or the ERAD pathway. Punctate SP-C is colocalized with KDEL-containing ER chaperones in both wild-type and homozygous mutant type II cells, suggestive of ER accumulation of SP-C. The fraction of ER accumulation of SP-C in the mutant type II cells was 0.84, while that of Golgi accumulation was 0.08, evaluated by confocal colocalization images. Furthermore, expression of CHOP, a transcription factor related to cell death during ER stress, increased in homozygous mutant lungs after birth, suggesting that mutant lung tissue might be suffering from ER stress. Although endosomal/lysosomal degradation of SP-C could not be excluded, these data suggested that, in homozygous mutant-BiP type II cells, misfolded SP-C might accumulate in the ER and be degraded by the ERAD pathway.

SP-C deficits are related to acute and chronic infant lung diseases in humans (Lahti et al., 2004). Furthermore, mutations in proSP-C have been correlated with chronic interstitial pneumonia (Beers & Mulugeta, 2005). ProSP-C is an type II integral membrane protein with structural homology to the amyloidogenic BRI family of proteins, which cause neurodegenerative dementia (Kim et al., 2002). Mutant proSP-C tends to misfold and may cause protein aggregation and ER stress (Beers & Mulugeta, 2005). Thus, aberrant quality control in the mutant-BiP type II epithelial cells might have resulted in proSP-C misfolding. Misfolded proSP-C might act to generate respiratory failure by causing ER stress in mutant type II cells in concert with decreased pulmonary surfactant levels (Mimura et al., 2007).

## 6. BiP, an ER chaperone, plays important roles in neuronal migration and stratification

The homozygous mutant BiP neonates died after birth due to respiratory failure (Mimura et al., 2007). Besides that the mutant BiP mice displayed disordered layer formation in the cerebral cortex and cerebellum (Mimura et al., 2008).

### 6.1 Defective neocortical layer formation in the mutant BiP mice

The homozygous mutant BiP neonates moved, responded to painful stimuli, but were significantly smaller than the wild-type. Among the various organs, the mutant brain, including the cerebral cortex and cerebellum, was substantially smaller than that in wild-type mice, suggesting that the brain was particularly affected by the BiP mutation. In fact, the neocortical stratification at embryonic day 18 (E18), as observed with the hematoxylin-eosin staining, was defective in the mutant BiP mice. The mutant brain had a relatively high density of neurons in neocortical layer I in contrast to a low density of neuronal arrangement in the control.

Cortical neurogenesis occurs in the ventricular zone, and the new neurons migrate through other neurons to the marginal zone and then move to their final destination during embryogenesis. To further investigate the defect in layer formation during neocortical development, birth date analysis of the neocortical neurons was carried out by BrdU labeling. The results showed that in the mutant brain the earlier-born neurons reached the superficial layer and remained there, and that the later-born neurons did not reach the upper layer, remaining in the lower layer. The mutant BiP mice exhibited an outside-in pattern of neocortical layer formation in contrast to the inside-out pattern in the control (Caviness, 1982), indicating that the neocortical layer formation was impaired.

### 6.2 Mutant BiP mice have reduced expression of reelin

The above findings suggested that aberrant neocortical formation was due to the defects in layer formation, like a deficiency in reelin signaling in a *reeler* mutant malformation (Falconer, 1951; D'Arcangelo et al., 1995). Indeed, analysis of embryonic cerebral neocortex revealed significantly reduced reelin expression by Western blot and immunoreactivity in the superficial layer I of the mutant BiP mice. These results are consistent with the fact that reelin is a secretory protein that may interact with BiP in the ER.

Reelin is a large secreted glycoprotein (D'Arcangelo et al., 1995) produced by some cortical neurons such as Cajal-Retzius (CR) cells in the marginal zone during development. Reelin mediates cortical laminar formation through the binding to very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor type 2 (ApoER2), leading to the phosphorylation of an adaptor molecule, Dab1, on cortical neurons (D'Arcangelo et al., 1999; Tissir and Goffinet, 2003). In *reeler* mice deficient in the reelin gene (D'Arcangelo et al., 1995), the cortical neurons lack the ability to localize properly, and settle inside the earlier migrating neurons (Caviness, 1982).

Because *reeler* malformation is also well documented in the cerebellum with regard to the migration defect of Purkinje cells (Yuasa et al., 1993), the structure of the cerebellum was examined at E18. The growth of the mutant cerebellum was significantly retarded, examined by the staining with DNA dye. Though the external granular layer (EGL) was formed in the both genotypes, the development of EGL migrating tangentially from the rhombic lip was

significantly retarded in the mutant BiP mice. A large number of Purkinje cells remained in the subcortical region in contrast to the cortical arrangement of Purkinje cells in the control. Hippocampal layer formation showed little defect in the mutant BiP mice.

The structure of the superficial layer of the neocortical primordium was further examined by double immunohistochemical labeling for both reelin and calretinin. Calretinin-immunopositive neurons, corresponding to CR cells in the neocortical primordium, were found in the superficial layer of the mutant BiP mice, but their number was significantly reduced and reelin immunoreactivity was barely detected, in contrast to the localization of reelin immunoreactivity in the calretinin-positive neurons in superficial layer I of the wild-type mice. Some of calretinin-immunopositive CR cells of the mutant neocortex appeared in a disorganized scattered pattern other than the marginal zone. This finding was confirmed by in situ hybridization histochemistry of the neocortical primordium by using *reelin* cRNA probe as the marker for CR cells. The cells positive for *reelin* mRNA formed the thin superficial layer in the wild-type mice. In the contrast, the cells positive for *reelin* mRNA were scattered in the upper layer of the neocortical primordium of the mutant BiP mice. These findings of in situ hybridization histochemistry corresponded well with those of calretinin-immunoreactive cells. Furthermore, these findings indicated that the transcription of *reelin* gene took place in a similar degree both in the mutant and wild-type mice, but the reelin protein was significantly reduced in the CR cells of the mutant.

While this mouse had features of a *reeler* mutant phenotype such as an outside-in pattern of neocortical layer formation and the migration defect of Purkinje cells in cerebellum, it also had other phenotypes in the brain, which were distinct from the *reeler* phenotype. These included the reduction in the size of the whole brain and the apparent scattering of reelin- and calretinin-positive neurons throughout the cortex. This is not surprising since BiP likely has a multitude of substrates significant for brain development.

### 6.3 Reelin secretion is impaired in the mutant BiP brain

Embryonic cerebral neocortex revealed significantly reduced reelin expression by Western blot in the homozygous mutant BiP, consistent with histological observations. Although VLDLR expression was equivalent between wild-type and mutant BiP cortex, dephosphorylated Dab1 accumulated in the mutant BiP brain, indicating that the reelin signaling pathway was inactivated there. The reelin deficiency was not a consequence of reduced transcription, because *reelin* mRNA expression did not differ in the control and mutant brains, consistent to the in situ hybridization experiment. The expression of *BiP* mRNA as well as CHOP protein was enhanced in the mutant brain, suggesting that the mutant brain suffered from ER stress.

Mutant BiP might impair the folding of reelin, leading to its degradation by the ERAD pathway or to its secretion as an immature form from the CR cells due to an escape from ER quality control. To test this possibility, we used primary neurons derived from embryonic brain and found a significant decrease in reelin secretion by the homozygous mutant BiP neurons compared with wild-type or heterozygous neurons. To investigate whether the homozygous mutant BiP neurons maintained their responsiveness to reelin stimulation, we incubated primary neurons with conditioned culture medium containing a several-fold physiological level of reelin secreted by 293T cells transiently transfected with reelin cDNA. Exogenous reelin seemed to be active on the homozygous neurons, leading to the activation of the reelin signaling pathway, as demonstrated by a reduced amount of Dab1 expression

and an increased amount of phospho-Dab1 expression. On the other hand, reelin signaling pathway in the heterozygous mutant cortical neurons seemed to be constitutively active with endogenous reelin even without exogenous reelin stimulation. Thus, Dab1 expression and phosphorylation were rather unchanged in the heterozygous mutant. These results suggested that impaired secretion of reelin by the CR cells rather than defective responsiveness in the cortical neurons might be responsible for the neurological phenotype of *reeler* mutant-like malformation in the mutant BiP mice. Thus, impaired retrieval of BiP might promote the degradation of misfolded proteins by the ubiquitin/ proteasome pathway. In fact, ubiquitinated proteins accumulated in the mutant cerebrum.

#### **6.4 BiP may enhance the folding of reelin**

Mutant BiP was detected in the ER (Mimura et al., 2007), but a significant fraction was also secreted from cells because of the lack of the retrieval motif (KDEL). We examined the subcellular localization of reelin to establish its relationship with mutant and wild-type BiP. Reelin co-localized with mutant BiP in the ER in primary neurons derived from heterozygous mutant BiP embryos; this was also the case in cortical neurons in the homozygous mutant postnatal brain where the expression of reelin was reduced. To obtain further insight into the interaction of BiP and reelin, we performed co-transfection experiments in HeLa cells. Co-expression of reelin and the wild-type BiP, but not the mutant BiP lacking the KDEL sequence, greatly enhanced the expression of reelin protein (reelin mRNA levels were equivalent in the two transfections). These results suggested that BiP promoted the folding of reelin.

Although the folding, intracellular transport and oligomerization of reelin have not been characterized in detail, we found that reelin protein expression was impaired in the mutant BiP mice, indicating that BiP might play a role in the maturation of reelin. Furthermore, we found that the expression of *BiP* mRNA and CHOP protein was enhanced in the mutant brain, suggesting that the mutant brain might have suffered from ER stress. We speculate that the folding of reelin protein might be vulnerable to impaired quality control in the ER and the post-ER compartments of mutant CR cells.

The mutant BiP mice revealed that a physiological increase in the production of reelin and surfactant proteins in dedicated secretory cells like CR cells and alveolar type II cells during neonatal periods may require BiP and a proper folding capacity in the ER. Neuronal migration and stratification may be sensitive to environmental insults such as viral infection, hypoxia and ischemia that perturb ER functions (Mimura et al., 2008).

### **7. BiP modulates the development of morphine antinociceptive tolerance**

Heterozygous mutant BiP mice produce pulmonary surfactant and reelin, and grow to be apparently normal adults. However, they may be potentially sensitive to ER stress. Morphine is a potent analgesic, but the molecular mechanism for tolerance formation after repeated use is not fully understood. We tested the thermal antinociceptive effect of morphine on the heterozygous mutant BiP mice in order to elucidate physiological processes that were sensitive to BiP functions (Dobashi et al., 2010).

#### **7.1 Morphine tolerance**

Opioids are potent analgesics that are widely used to control acute and chronic pain (Somogyi et al., 2007). Although repeated administration of opioids, particularly morphine, induces



tolerance that reduces the effectiveness of the analgesic, the precise molecular mechanism for the development of tolerance remains uncertain. Opioids bind to the mu opioid receptor (MOR) to activate various signaling molecules through heterotrimeric guanine nucleotide-binding proteins (G proteins), leading to a decrease in neuronal excitability by the inhibition of voltage-dependent calcium channels and the activation of inwardly rectifying potassium channels (Dickinson et al., 2000). Activation of MOR also induces the phosphorylation of MOR by G-protein-coupled receptor kinases (Johnson et al., 2005). Phosphorylated MOR is recognized by arrestins (Bohn et al., 1999), and internalized by clathrin-coated vesicles. The transient uncoupling of MOR from signaling pathways due to the phosphorylation and intracellular trafficking of MOR causes opioid desensitization. Most of the internalized MORs return to the cell surface, resulting in resensitization (Zollner et al., 2008).

Chronic morphine tolerance may be derived from adaptations in the intracellular signal transduction of post-MOR activation, as morphine does not induce effective MOR phosphorylation and internalization (Finn and Whistler, 2001). Persistent MOR activation may alter signal transduction, including changes in MOR-coupled G proteins from  $G_{i\alpha}$  to  $G_{s\alpha}$  (Chakrabarti et al., 2005), increased activity of protein kinase C (Granados-Soto et al., 2000), and the upregulation of N-methyl-D-aspartate receptor signaling (Trujillo and Akil, 1991). These changes may contribute to the development of morphine tolerance. Chronic morphine treatment also activates the cyclin-dependent kinase 5 and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) signaling pathway, while the inhibition of them diminishes morphine tolerance and restores analgesia in rats (Parkitna et al., 2006). GSK3 $\beta$  is expressed ubiquitously and is one of the central molecules in intracellular signal transduction (Grimes and Jope, 2001). It may play an important role in diverse physiological and pathological states (Jope et al., 2007).

### **7.2 Morphine tolerance is attenuated in mice expressing a mutant BiP**

We examined whether BiP might affect morphine analgesia using heterozygous mutant BiP mice. We evaluated morphine-induced antinociception by measuring response latencies in a hot plate test. Morphine tolerance was induced by intraperitoneal morphine injection twice a day for 5 consecutive days. We performed hot plate tests at the first and the tenth morphine treatments. The response latencies of the mutant BiP mice and their wild-type littermates before morphine treatment on day 1 were not significantly different. The time courses of response latencies of the both groups at the first morphine treatment on day 1 were almost similar, and both latencies reached the 60 s cut-off point 30 min after injection. Even just before the tenth morphine treatment on day 5, the response latencies of the both groups were not significantly different. These results indicated that the mutant BiP mice had normal sensory transmission and analgesia. After the tenth morphine treatment on day 5, the response latencies of the wild-type mice were significantly reduced, indicating that morphine tolerance had developed. However, the response latencies of the mutant BiP mice after the tenth morphine treatment were significantly longer than those of their wild-type littermates at 30, 45 and 60 min after injection. These results showed that the mutant BiP mice were impaired in the development of morphine tolerance (Dobashi et al., 2010).

### **7.3 Inhibition of GSK3 $\beta$ signaling is associated with the prevention of morphine tolerance**

Since alterations in the intracellular trafficking of MOR might affect opioid analgesia, we examined the effect of the mutant BiP with the KDEL sequence deleted on the surface

expression of MOR. MEFs from the wild-type and homozygous mutant embryos (Mimura et al., 2007) were transfected with a myc-tagged MOR. DAMGO, a selective peptidergic MOR ligand induces the internalization of MOR but morphine does not internalize MOR. We found the surface expression of MOR in both wild-type and mutant MEFs by confocal laser microscopy. While DAMGO induced the internalization of MOR in both types of MEFs, MOR remained on the cell surface upon morphine treatment, suggesting that the mutant BiP did not affect the transport of MOR.

Then, we speculated that the UPR signaling might attenuate the MOR signaling, which might cause the development of morphine tolerance. GSK3 $\beta$  is one possible candidate molecule that may play key roles in both the UPR and MOR signaling pathways. Recently the inhibition of GSK3 $\beta$  by the specific inhibitors SB216763 and (2'Z, 3'E)-6-bromoindirubin-3'-oxime was shown to diminish the development of morphine tolerance in rats after chronic intrathecal morphine treatment (Parkitna et al., 2006). The kinase activity of GSK3 $\beta$  is regulated by its phosphorylation status. Phosphorylation of residue Ser9 inactivates the activity, whereas dephosphorylation of Ser9 and phosphorylation of Tyr216 enhance the activity (Grimes and Jope, 2001).

We evaluated the phosphorylation status of GSK3 $\beta$  in the brain stems of wild-type and heterozygous mutant BiP mice using specific antibodies against phosphorylated Tyr216 GSK3 $\beta$  and phosphorylated Ser9 GSK3 $\beta$ . After chronic morphine injection intraperitoneally for 5 days, the wild-type mice developed morphine tolerance, whereas the mutant BiP mice remained less tolerant to morphine. Because we injected morphine intraperitoneally, both spinal and supraspinal neurons were supposed to be affected. Neurons with MOR expression in the periaqueductal gray (PAG) matter contribute to morphine tolerance (Yaksh et al., 1976; Bagley et al., 2005; Morgan et al., 2006). With repeated morphine treatment, the mutant BiP brain stems showed low levels of phosphorylation of Tyr216 in GSK3 $\beta$ , in contrast to the prominent phosphorylation in wild-type mice by Western blot. After chronic morphine injection intraperitoneally for 5 days in both types of mice, the brains were sectioned and double-immunostained with antibodies raised against MOR and tyrosine-phosphorylated GSK3 $\beta$ . MOR-immunopositive neurons in the PAG region of wild-type brains showed more enhanced expression of tyrosine-phosphorylated GSK3 $\beta$  significantly than those in the mutant BiP brains.

These observations suggested that chronic MOR stimulation by repetitive morphine injection might activate GSK3 $\beta$ , leading to the development of morphine tolerance. Mice with the mutant BiP might be defective in the activation of GSK3 $\beta$ . Furthermore, we examined embryonic brains of homozygous mutant BiP mice which did not express the wild-type BiP. Western blot revealed prominent phosphorylation at Ser9 of GSK3 $\beta$  and less phosphorylation at Tyr216 in the homozygous mutant BiP brain compared to those in the wild-type brain. These results suggested that the mutant BiP lacking the KDEL sequence might attenuate the activation of GSK3 $\beta$  in vivo (Dobashi et al., 2010).

#### **7.4 Chemical chaperone attenuates the development of morphine tolerance**

In order to confirm that an ER chaperone mediates the development of morphine tolerance, we examined the effect of a chemical chaperone on morphine tolerance. Tauroursodeoxycholic acid (TUDCA) is a derivative of endogenous bile acids that is thought to increase ER folding capacity and suppresses the expression of BiP (Xie et al., 2002; Ozcan et al., 2006). We administered TUDCA together with morphine twice a day for 5

days in wild-type mice, and hot plate tests were performed at the first and the tenth treatments. The response latencies of the mice receiving both TUDCA and morphine were significantly longer than those of control mice with morphine alone after the tenth treatment. Thus, TUDCA prevented the development of morphine tolerance, suggesting a mechanistic relationship between an ER chaperone and morphine tolerance.

These results suggested a novel function of BiP on the development of morphine tolerance *in vivo*. The modulation of morphine analgesia by TUDCA revealed a potential clinical application of chemical chaperones that could modulate ER functions for the prevention of morphine tolerance (Dobashi et al., 2010).

## **8. Dysfunction of the ER chaperone, BiP, may accelerates the organic injury with aging**

Heterozygous mutant BiP mice express both the wild type BiP and the mutant BiP, in which the UPR is not induced in the steady state. However, it is possible that potential vulnerability to ER stress may exist in the mutant BiP mice, resulting in organic injury during chronic stress such as aging (Kimura et al., 2008).

### **8.1 Heterozygous mutant BiP knock-in mice developed marked tubular-interstitial lesions with aging**

We found that some aged mutant BiP mice developed a severe tubular-interstitial lesion which consisted of tubular atrophy, tubular luminal dilatation and interstitial fibrosis. Mutant BiP mice over 80 weeks of age showed more severe tubular-interstitial lesions than age-matched wild type mice.

Proteinuria plays a key role in the tubular cell injury involved in human kidney disease (Remuzzi & Bertani, 1998). We used the bovine serum albumin (BSA)-overload proteinuria mouse model to clarify whether the tubular cell injury induced by proteinuria was associated with the tubular-interstitial lesions observed in aged heterozygous mutant BiP mice. BSA was injected into young mutant BiP mice (25-40 weeks of age) and control wild type mice intraperitoneally 5 days per week for 6 weeks after the uninephrectomy. While the mutant BiP mice maintained renal tissue apparently as normal as the wild type mice, the BSA treatment caused severe tubular-interstitial injury only in the mutant BiP mice. The mutant BiP mice with BSA overload had a significantly higher tubular damage score than the wild-type mice. Furthermore, the serum creatinine level of the BSA-treated mutant BiP mice was also significantly higher than that of the wild-type mice, indicating that proteinuria impaired the renal function of the mutant mice. No significant difference in creatinine level was observed between the control wild type mice and the mutant BiP mice without BSA treatment.

In order to determine whether the differences in renal injury with BSA treatment between heterozygous mutant BiP mice and wild type mice could be due to different levels in urinary protein excretion, we examined the urinary protein of the two different groups. After the uninephrectomy, BSA (10 mg/ body weight(g)) was injected intraperitoneally into the mice once a day for 7 days and urinary protein excretion was examined on day 8. We did not find any significant differences in urinary protein excretions between the mutant BiP mice and wild type mice. Thus, these results suggested that the tubular-interstitial tissue of the mutant BiP mice was more sensitive to proteinuria than that of the wild-type mice.

### **8.2 Caspase-12 activation and tubular cell apoptosis occurred in the kidneys of BSA-treated heterozygous mutant BiP knock-in mice**

BSA is reported to cause apoptosis in a murine proximal tubular cell line through a caspase-12 dependent apoptotic-pathway induced by ER stress (Ohse et al., 2006). In order to detect apoptosis in the BSA-treated tubular-interstitial lesions of heterozygous mutant BiP mice, we performed TUNEL staining. The number of TUNEL positive tubular cells in BSA-treated mutant BiP mice was significantly higher than that in the wild type. We also found the activation of caspase-12 in the BSA-treated mutant kidney by Western blot that showed the cleavage of caspase-12. Taken together, these results suggested that the renal tissue of the mutant BiP mice suffered from ER stress and was sensitive to proteinuria, which might cause tubular-interstitial lesions and apoptosis through ER stress pathway.

The involvement of ER stress and its downstream caspase-12 dependent apoptotic pathway on tubular cell injury induced by proteinuria have been reported (Ohse et al., 2006). Aged heterozygous mutant BiP mice showed glomerular lesion consisting of mesangial matrix increase and glomerular sclerosis. While we could not exclude the contribution of the glomerular lesion, high susceptibility to proteinuria due to potential ER dysfunction might cause the tubular-interstitial lesion of aged heterozygous mutant BiP mice. It is possible that potential vulnerability to ER stress might exist in the tubular cells of heterozygous mutant BiP mice and resulted in severe tubular injury during periods of chronic stress such as aging and proteinuria. Tubular cells are most sensitive to ER stress when mice are injected with tunicamycin that disturbs protein glycosylation in the ER and causes ER stress, which results in acute renal tubular necrosis (Zinszner et al., 1998). Consistently, we found caspase-12 activation in the kidneys of BSA-treated mutant-BiP mice, indicating that the ER stress pathway might have been involved in the BSA-mediated tubular-interstitial injury (Kimura et al., 2008).

## **9. Conclusion**

Quality control in the early secretory pathway is a ubiquitous mechanism for adapting to ER stress, and the KDEL receptor and BiP are essential components of this system. However, at various developmental stages, some cell types may require specific quality control systems and chaperones. Mutant mouse models have revealed that the UPR plays a vital role during development by increasing protein synthesis as needed in dedicated secretory cells, such as pancreatic beta cells (Scheuner et al., 2001), plasma cells (Reimold et al., 2001) and hepatocytes (Reimold et al., 2001). Inadequate adaptation to these types of physiological demands may lead to diverse diseases.

We produced knock-in mice expressing a mutant BiP lacking the retrieval sequence to examine the effects of defects in stress response in the secretory pathway without completely eliminating BiP function (Mimura et al., 2007) - as would be the case in BiP knockout mice (Luo et al., 2006). Mutant BiP mice have a distinct phenotype, as is the case for mice lacking other ER molecular chaperones. Hsp47 is responsible for collagen biosynthesis, and Hsp47 knockout mice die on E11.5 (Nagai et al., 2000). Calreticulin and calnexin participate in glycoprotein folding in the ER. Calreticulin knockout mice are embryonic lethal and display defective cardiac development (Mesaeli et al., 1999). Calnexin knockout mice die during the early postnatal period, between birth and three months of age. These mice exhibit motor disorders due to a loss of large myelinated nerve fibers (Denzel et al., 2002). Mutant BiP predominantly affected dedicated secretory cells, such as alveolar type

II cells and Cajal-Retzius cells, in which active secretion is particularly important. Putative impairment of protein folding in these mutant cells probably caused the observed respiratory failure and neurological disorders.

Deletion of the retrieval sequence from BiP, and the consequent lack of mutant BiP recycling by the KDEL receptor, could have two possible effects. First, the folding environment in the ER and post-ER may be impaired. Mutant BiP is functional as long as it remains in the ER. Therefore, constitutive activation of the UPR could compensate for the altered folding environment by producing mutant BiP in quantities sufficient for cell survival. However, quality control in post-ER compartments may also be affected. Proper ER-to-Golgi transport and subsequent ER retrieval of proteins and lipids are thought to contribute to quality control (Hammond and Helenius, 1994; Yamamoto et al., 2001). In this regard, the folding (and therefore function) of a pulmonary surfactant protein, proSP-C, and reelin may depend on proper ER retrieval of BiP via the KDEL receptor. Second, signal transduction during UPR could be affected. In addition to retrieval, the recognition of the KDEL sequence of BiP (and other KDEL proteins) by the KDEL receptor leads to signal transduction. The activation of the KDEL receptor may trigger subsequent activation of signaling molecules such as ARF1GAP (Yamamoto et al., 2001), src (Bard et al., 2003), protein kinase A (Cabrera et al., 2003), and mitogen-activated protein kinases (Yamamoto et al., 2003). Chronic morphine administration may cause altered signal transduction through persistent MOR activation. It would be possible that the crosstalk between MOR analgesic signal transduction and BiP-KDEL receptor signal transduction may affect morphine tolerance formation in the mutant BiP mice (Dobashi et al., 2010).

The heterozygous mutant BiP mice grew up to be adults and showed apparently normal organ development. There was no significant differences in life span between the wild type mice and the heterozygous mutant BiP mice. However, potential vulnerability to ER stress may exist in the mutant BiP mice, resulting in some organic injuries with aging as described above (Kimura et al., 2008). We are interested in the relationship between ER stress and the neural degeneration with aging. In this regard, several studies have suggested the possible role of reelin in the pathogenesis of human mental disorders such as schizophrenia, autism, bipolar disorder and Alzheimer's disease (Bothwell & Giniger, 2000; Tissir & Goffinet, 2003; Fatemi, 2005). Reelin and ApoE share ApoER2 on cortical neurons (D'Arcangelo et al., 1999), and ApoE inhibits reelin signaling by competing for binding to ApoER2. Interestingly, the E4 allele of ApoE increases the risk of developing sporadic forms of Alzheimer's disease. Because reelin signaling through ApoER2 in adult brains modulates synaptic plasticity and memory formation (Beffert et al., 2005), the defective reelin signaling pathway may contribute to the pathogenesis of adult mental disorders.

In the meantime, the persistent accumulation of misfolded proteins beyond the capacity of ER quality control causes ER stress, leading to cellular dysfunction and cell death (Kaufman, 2002; Kopito & Ron, 2000). This process is thought to cause human mental disorders such as neurodegenerative diseases including Alzheimer's disease (Katayama et al., 1999) and Parkinson's disease (Imai et al., 2001), bipolar disorders (Kakiuchi et al., 2003), and ischemic neuronal injury (Tajiri et al., 2004). The involvement of impaired BiP function in neurodegenerative diseases has been reported in a mouse model where the disruption of SIL1, a co-chaperone of BiP, causes protein accumulation and neurodegeneration (Zhao et al., 2005). Thus, reelin signaling and ER quality control may be related to the pathogenesis of adult mental disorders, as seen in reeler mutant-like cerebral malformation in mutant BiP neonates (Mimura et al., 2008).

Our results suggest that ER stress would be a promising therapeutic target with which to combat chronic organ injuries. In order to treat ER stress related diseases, two kinds of strategy will be effective: the promotion of protein folding in the ER and the inhibition of an ER stress induced apoptotic pathway. Indeed, the administration of chemical chaperones that promote protein folding in the ER has been reported to be effective in treating type2 diabetes, which has been speculated to be caused by ER stress after experiments using a mouse model (Ozcan et al., 2006). We also showed that a chemical chaperone prevented the development of morphine tolerance (Dobashi et al., 2010). The heterozygous mutant BiP knock-in mice used in our experiments will be a suitable tool for investigating the relationship between ER stress and organ dysfunctions with aging.

## 10. Acknowledgements

I thank Drs. Victor W. Hsu and Haruhiko Koseki for helpful suggestions. I also thank Katsushi Yamamoto, Rika Fujii, Hiromichi Hamada, Naoya Mimura, Keita Kimura, Serabi Tanabe, Tamae Dobashi and Hisayo Jin as laboratory members. This work was supported by Grants-in-Aids for Science Research from the Ministry of Education, Culture, Sports, Science and Technology to T.A.

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## **Applications and Experiences of Quality Control**

Edited by Prof. Ognyan Ivanov

ISBN 978-953-307-236-4

Hard cover, 704 pages

**Publisher** InTech

**Published online** 26, April, 2011

**Published in print edition** April, 2011

The rich palette of topics set out in this book provides a sufficiently broad overview of the developments in the field of quality control. By providing detailed information on various aspects of quality control, this book can serve as a basis for starting interdisciplinary cooperation, which has increasingly become an integral part of scientific and applied research.

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Tomohiko Aoe (2011). Quality Control in the Early Secretory Pathway Plays Significant Roles in vivo, Applications and Experiences of Quality Control, Prof. Ognyan Ivanov (Ed.), ISBN: 978-953-307-236-4, InTech, Available from: <http://www.intechopen.com/books/applications-and-experiences-of-quality-control/quality-control-in-the-early-secretory-pathway-plays-significant-roles-in-vivo>

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