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ORIGINAL PAPER

Sublethal endoplasmic reticulum stress caused by the mutation of immunoglobulin heavy chain-binding protein induces the synthesis of a mitochondrial protein, pyrroline-5-carboxylate reductase 1

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Abstract Most human neurodegenerative diseases are sporadic and appear later in life. Aging and neurodegeneration are closely associated, and recent investigations reveal that endoplasmic reticulum (ER) stress is involved in the progression of these features. Immunoglobulin heavy chain-binding protein (BiP) is an ER chaperone that is central to ER functions. We produced knock-in mice expressing a mutant BiP that lacked the retrieval sequence to elucidate the effect of a functional defect in an ER chaperone in multicellular organisms. The homozygous mutant BiP mice died within several hours after birth because of respiratory failure with an impaired biosynthesis of pulmonary surfactant by alveolar type II cells. The heterozygous mutant BiP mice grew up to be apparently normal adults, although some of them revealed motor disabilities as they aged. Here, we report that the synthesis of a mitochondrial protein, pyrroline-5-carboxylate reductase 1 (PYCR1), is enhanced in the brains of homozygous mutant BiP mice. We performed a two-dimensional gel analysis followed by liquid chromatography-tandem mass spectrometry. PYCR1 was identified as one of the enhanced proteins. We also found that sublethal ER stress caused by tunicamycin treatment induced the synthesis of PYCR1 in murine fibroblasts. PYCR1 has been shown to be related to the aging process. Mutations in the PYCR1 gene cause cutis laxa with progeroid features and mental retardation. These findings suggest a pathophysiological interaction between ER stress and a mitochondrial function in aging.

Keywords Aging · Chaperone · ER stress · Mitochondria · UPR

Introduction

The endoplasmic reticulum (ER) plays a major role in the protein synthesis of secretory pathways (Ellgaard and Helenius 2003). ER molecular chaperones and folding enzymes such as immunoglobulin heavy chain-binding protein (BiP), calnexin, and protein disulfide isomerase (PDI) facilitate the correct folding or degradation of these newly synthesized proteins as well as misfolded proteins (Pfaffenbach and Lee 2011). The accumulation of misfolded proteins in the ER causes ER stress and induces the unfolded protein response (UPR) that expands the capacity of quality control (Ron and Walter 2007). Further ER stress can cause cellular dysfunction and cell death, resulting in a diversity of human disorders such as neurodegenerative diseases (Jin et al. 2014; Roussel et al. 2013) and cardiomyopathy (Hamada et al. 2004). Besides pathological states, the UPR also plays an essential role in the physiological development of multicellular organisms. The UPR accommodates the capacity of protein synthesis in secretory cells to cope with the expanding physiological demand, as reported in alveolar type II cells (Mimura et al. 2007), Cajal–Retzius cells (Mimura et al. 2008), pancreatic beta cells (Harding et al. 2001), plasma cells (Reimold et al. 2001), and hepatocytes (Wang et al. 2012).

Mammalian ER luminal chaperones like BiP have a carboxyl-terminal Lys-Asp-Glu-Leu (KDEL) amino acid sequence, which is recognized by the KDEL receptor in post-ER

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compartments (Lewis and Pelham 1992). ER chaperones and the KDEL receptor are sorted into the transport vesicles coated with coat protein I complex and retrieved to the ER (Orci et al. 1997). Yeast BiP (Kar2p) is essential for survival, whereas the deletion of the retrieval sequence (in yeast: His-Asp-Glu-Leu) is dispensable because the UPR is activated, compensating for the loss of the chaperone in the ER (Beh and Rose 1995). In mammals, the complete depletion of BiP has been shown to have a lethal effect on early embryonic cells (Luo et al. 2006).

To elucidate the physiological processes that are sensitive to the retrieval of BiP during the development and adulthood in multicellular organisms, we previously produced knock-in mice expressing a mutant BiP in which the retrieval sequence was deleted by a homologous recombination. The homozygous mutant BiP mice died within several hours after birth because of respiratory failure with impaired biosynthesis of the pulmonary surfactant, especially surfactant protein C, by alveolar type II cells (Mimura et al. 2007). In addition, we found that the mutant BiP mice displayed a disordered layer formation in the cerebral cortex and cerebellum; this is a neurological phenotype of the *reeler* mutant-like malformation (D'Arcangelo et al. 1995). Consistent with the phenotype, Cajal–Retzius cells did not secrete reelin, and post-transcriptionally, its synthesis was markedly reduced (Mimura et al. 2008).

We evaluated the effect of sublethal ER stress because of the BiP mutation by analyzing the profile of protein expression. Here, we report that the synthesis of a mitochondrial protein, pyrroline-5-carboxylate reductase 1 (PYCR1), is enhanced in the brain of homozygous embryonic mutant BiP mice. We performed two-dimensional (2D) protein separations, followed by a liquid chromatography-tandem mass spectrometry (LCMS/MS) analysis. PYCR1 was identified as one of the enhanced proteins. We also found that sublethal ER stress caused by tunicamycin induced the synthesis of PYCR1 in murine embryonic fibroblasts (MEFs). PYCR1 has been shown to be related to the aging process. Mutations in the PYCR1 gene cause cutis laxa with progeroid features and mental retardation (Guernsey et al. 2009; Reversade et al. 2009). These findings show that sublethal ER stress affects the biosynthesis of a mitochondrial protein related to aging.

Materials and methods

Mutant BiP mice

All animal experimental procedures were in accordance with a protocol approved by the Institutional Animal Care Committee of Chiba University, Chiba, Japan. We used a homologous recombination to establish knock-in mice expressing BiP lacking the carboxyl-terminal KDEL sequence (Mimura et al. 2007). The missing KDEL sequence was replaced by a hemagglutinin (HA) tag. The mutant BiP mice were investigated on the compound genetic background between the 129/SvJ derived from R1 ES cells and C57BL/6J. The heterozygotes were intercrossed to generate homozygous mutants. This hybrid line was maintained by brother–sister mating for at least ten generations.

Cells and reagents

MEFs were prepared from 13.5-day-old embryos (Mimura et al. 2007). MEFs were grown in a complete medium that consisted of Dulbecco's modified Eagle's medium (Sigma Chemical Co., Irvine, UK) with 10 % fetal bovine serum, 2 mM glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin G at 37 °C in a 5 % CO_2 incubator. The following antibodies were used: a rabbit antiserum against CCAAT-enhancer-binding protein homologous protein (CHOP, sc-793, Santa Cruz Biotechnology, 1:500 dilution for Western blotting), a rabbit antiserum against PYCR1 (13108-8-AP, ProteinTech Group, 1:1000 dilution for Western blotting, 1:100 dilution for immunofluorescence), a rabbit antiserum against cleaved caspase 3 (#9661, Cell Signaling, 1:500 dilution for Western blotting), a rabbit antiserum against glycogen synthase kinase 3β (GSK3β, sc-9166, Santa Cruz Biotechnology, 1:100 dilution for immunofluorescence), a mouse mAb against activating transcription factor 6 (ATF6) α (37-1, 73-505, Bio Academia, 1:500 dilution for Western blotting), a mouse mAb against γ-tubulin (T6557, Sigma, 1:1000 dilution for Western blotting), a mouse mAb against BiP (KDEL sequence, ADI-SPA-827, Enzo life science, 1:1000 dilution for Western blotting, 1:100 dilution for immunofluorescence), a mouse mAb 15E6 against HA tag (kindly provided by VW Hsu, Boston, MA, culture soup, 1:2 dilution for Western blotting, 1:10 dilution for immunofluorescence), a mouse mAb against cytochrome *c* oxidase IV (Cox IV, ab33985, abcam, 1:100 dilution for immunofluorescence), a donkey anti-mouse IgG Alexa Fluor488 (Invitrogen, 1:100 dilution for immunofluorescence), and a donkey anti-rabbit IgG Alexa Fluor555 (Invitrogen, 1:500 dilution for immunofluorescence). Tunicamycin was purchased from Nacali Tesque. Hoechst 33,258 (10 mM, B-1155, 1:500 dilution for immunofluorescence) was purchased from Sigma-Aldrich. An ATF6 inhibitor (0.2 mM, AEBSF; 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, A8456), an IRE1 inhibitor (60 μM, STF-083010, SML-0409) and a translation inhibitor (1 μg/ml, cycloheximide) were purchased from Sigma-Aldrich.

2D gel and LCMS/MS analysis

We performed 2D gel electrophoresis using a ReadyPrep 2D starter kit (Bio-Rad) based on the manufacturer's instruction. The mice were deeply anesthetized with an intraperitoneal

injection of pentobarbital (Dainippon Sumitomo Pharma). Mouse brains were obtained and homogenized by sonication (UR-20P, TOMY) in a buffer containing 8 M urea, 2 % CHAPS, 50 mM dithiothreitol, 0.2 % (*w*/*v*) Bio-Lyte 3/10 ampholyte, and 0.001 % Bromophenol Blue. Twenty micrograms of protein samples were applied to isoelectric focusing (IEF) using ReadyStrip IPG strips (7 cm, pH 5–8). The electrophoresis was run according to the manufacturer's instruction. The IPG strips preceded running the second dimension of SDS-PAGE with 8 % gel or 12 % gel. Then, the 2D gels were fixed with 50 % ethanol and stained by silver staining. The significantly dense spot derived from the homozygous mutant brain was excised from the gel and analyzed by LCMS/MS (APRO Science, Tokushima, Japan).

Western blotting

The mouse brains were obtained as above and homogenized by sonication (UR-20P, TOMY) in a buffer containing 0.4 % (*w*/*v*) Nonidet P-40, 0.2 % *N*-lauroylsarcosine, 10 mM Tris/HCl pH 8.0, 30 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 30 μg/ml *N*-acetyl-L-leucinal-L-lecinal-L-norleucinal, and 1 mM sodium orthovanadate (Sigma Chemical). Cultured cells were washed twice with ice-cold PBS and then homogenized in the same buffer. The lysates were centrifuged, and the supernatants were resuspended in the SDS-PAGE sample buffer and then separated by SDS-PAGE under reducing conditions. The proteins were transferred from the gels to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp.), and Western blotting was done as previously described (Hamada et al. 2004). Imaging was obtained by LAS-1000 and Image Gauge software (Fuji Photo Film Co. Ltd.).

Immunofluorescence microscopy

Cells on the coverslips were fixed in cold methanol for 60 min at −20 °C and then processed as previously described (Yamamoto et al. 2001). The stained cells were examined by fluorescence microscopy (Axiovert 200 M, Carl Zeiss, Oberkochen, Germany). Immunolocalization was observed by a fluorescence microscope using FITC/rhodamine filters with Plan-Neofluar 20× NA 0.50 and Plan-Neofluar 40× NA 0.75 objectives. Immunofluorescence images were captured with a digital camera (AxioCam MRm, Carl Zeiss). The brightness and contrast were optimized by AxioVision 4.4 software (Carl Zeiss).

Detection of mRNA

mRNAs were extracted from MEFs, and cDNAs were synthesized as previously described (Mimura et al. 2007). RT-PCR analysis was performed with the following primers: 5′-AAGATGGCAGGCTTGTGGAGCA-3′ and 5′-CAGAGCATCCAGGGCTGTGAAA -3′ for PYCR1 (MP212260, OriGene Technologies), and 5′-ATGGGGTGAGGCCGGTGCTG-3′ and 5′-CTTGATGTCATCATACTTGG-3′ for GAPDH.

siRNA

siRNA oligonucleotides for PYCR1 (sc-93,719) and control (sc-37,007) were purchased from Santa Cruz Biotechnology. For siRNA uptake, the mutant BiP MEFs were transfected with siRNA oligonucleotide according to manufacturer's instructions. At 24-h post-transfection, the medium was changed to a complete medium and cells were incubated for 24 h. Then, cells were incubated for 12 h with tunicamycin (5 μg/ml).

Results

The homozygous mutant BiP mice were born at the expected Mendelian ratio, and they died within 1 day after birth because of respiratory failure (Mimura et al. 2007). They moved, responded to painful stimuli, but appeared pale and were significantly smaller than wild-type mice. Among the various organs, the mutant brain, including the cerebral cortex and cerebellum, was substantially smaller than in wild-type mice, suggesting that the brain was particularly affected by the BiP mutation (Mimura et al. 2008). In fact, we found that the mutant BiP mice displayed a disordered layer formation in the cerebral cortex and cerebellum. Like *reeler* mice, Cajal–Retzius cells in the mutant BiP mice did not secrete reelin (D'Arcangelo et al. 1995), and its expression was markedly reduced post-transcriptionally (Mimura et al. 2008). To investigate other factors sensitive to the BiP mutation in the brain, we analyzed embryonic brain samples from the homozygous mutant BiP mice and wild-type mice by 2D gel electrophoresis. Among the several protein spots, we focused on one particular spot that was consistently over-expressed in the mutant brain compared with that in the wild-type brain (Fig. 1, Spot 1). The electrophoretic spots, obtained from 2D gels, were analyzed by LCMS/MS. We found that the Spot 1 represented PYCR1. As a control, we identified another spot of which expressions were similar in both the mutant brain and the wild-type brain, which turned out to be cleaved caspase 3 (Fig. 1, Spot 2). We confirmed these findings by Western blotting using an anti-PYCR1 antiserum and an anti-cleaved caspase 3 antiserum, following 2D gel electrophoresis (Fig. 2).

Since PYCR1 is supposed to be a mitochondrial protein (Dougherty et al. 1992), we examined the expression of PYCR1 by immunofluorescence with an anti-PYCR1 antiserum in MEFs from wild type and the homozygous mutant BiP

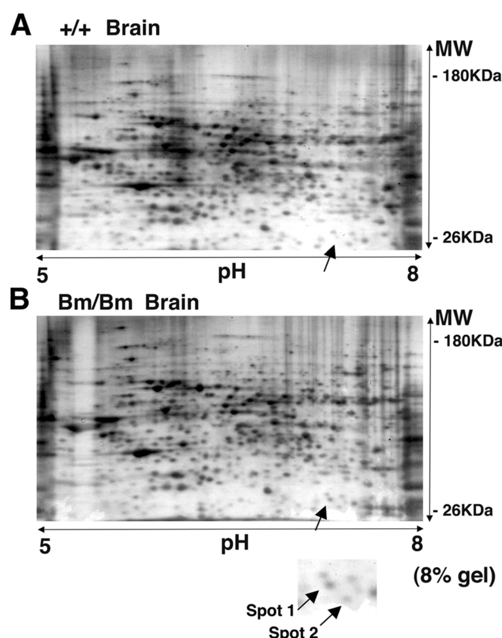

Fig. 1 Two-dimensional (2D) gel analysis for wild-type (+/+, **a**) and mutant BiP (*Bm/Bm*, **b**) embryonic brains. We performed 2D gel electrophoresis. The first dimension was done by isoelectric focusing at pH 5-8. The second dimension was done by SDS-PAGE with 8 % gel. Then, the 2D gels were fixed with 50 % ethanol and stained by silver staining. Spot 1 *arrowhead* in the homozygous mutant BiP embryonic brain (*Bm/Bm*) indicates the significantly dense spot, which turned out to be pyrroline-5-carboxylate reductase 1 (PYCR1). Spot 2 *arrowhead* shows another spot that expressed to the same extent in both types of mice, indicating cleaved caspase 3. The small panel shows the 2D gel around the spots at large magnification

mice. PYCR1 was co-stained with cytochrome *c* oxidase IV, the terminal oxidase in the mitochondrial electron transport system (Fig. 3, left panels). We found that PYCR1 was

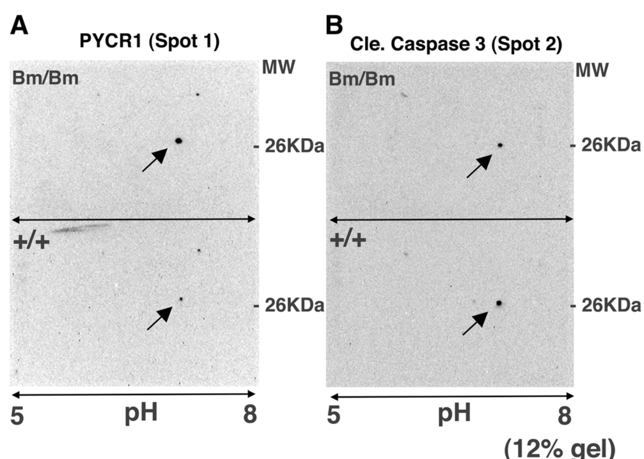

Fig. 2 Two-dimensional gel analysis for wild-type (+/+) and mutant BiP (*Bm/Bm*) embryonic brains, followed by Western blotting. The first dimension was done by isoelectric focusing at pH 5–8. The second dimension was done by SDS-PAGE with 12 % gel. The proteins were transferred from the gels to polyvinylidene fluoride membranes, and Western blotting was done with an anti-pyrroline-5-carboxylate reductase 1 (*PYCR1*) rabbit antiserum (**a**) and an anti-cleaved caspase 3 rabbit antiserum (**b**)

expressed in both types of cells and that the expression seemed to be enhanced by ER stress with a tunicamycin treatment, even in the wild type (Fig. 3). As a control, a staining with an anti-GSK3β antiserum revealed a cytoplasmic distribution (Fig. 3c). These observations support the fact that PYCR1 is localized to the mitochondrion.

We also examined the expression of BiP. The distribution of PYCR1 was different from that of the wild-type BiP and the mutant BiP (Fig. 4). The anti-BiP mAb recognizes the KDEL sequence. Thus, it also recognizes other ER chaperones with the KDEL sequence such as 94-kD glucose-regulated protein (GRP94) and PDI. The anti-HA mAb only recognizes the mutant BiP tagged with HA instead of the KDEL. While the distributions of the mutant BiP and PYCR1 were different from each other, a small portion of them seemed to be overlapped (Fig. 4b). That may be possible, since the ER and the mitochondrion have close relationships physically and functionally (de Brito and Scorrano 2010; Vannuvel et al. 2013).

We then examined the expression of PYCR1 by Western blotting in the wild-type and homozygous mutant BiP MEFs. We found that the synthesis of PYCR1 was a little in the resting state, while it was markedly enhanced in both types of MEFs by ER stress with a tunicamycin treatment that induced the synthesis of BiP, mutant BiP, and CHOP (Fig. 5). We performed semi-quantitative RT-PCR analysis for measuring PYCR1 mRNA level in the MEFs. ER stress by tunicamycin treatment enhanced the expression of PYCR1 mRNA, while that of GADPH remained stable (Fig. 6a). Inhibition of protein translation with cycloheximide during tunicamycin treatment suppressed the expression of PYCR1 protein, while that of tubulin remained stable (Fig. 6b). Thus, these results suggest that the transcription of PYCR1 during ER stress is up-regulated, resulting in the enhanced expression of PYCR1 protein.

ER stress signaling is transduced by the activation of inositol-requiring kinase-1 (IRE1), PKR-like ER-associated kinase (PERK), and ATF6 (Bertolotti et al. 2000; Schroder and Kaufman 2005). To investigate the relationship between the UPR and the expression of PYCR1 further, we examined the expression of PYCR1 under ER stress with a tunicamycin treatment, partially inhibited by an IRE1 inhibitor or an ATF6 inhibitor (Fig. 7). An ATF6 inhibitor, AEBSF, reduced the expression of PYCR1 in the mutant BiP MEFs, whereas it preserved the expression of mutant BiP. Thus, the induction of PYCR1 seems to rely mainly on the ATF6 pathway during the UPR.

We tried the knockdown experiment on PYCR1 mRNA to elucidate whether the expression of PYCR1 protein during the UPR is cytoprotective or pro-apoptotic. siRNA for PYCR1 in the mutant BiP MEF treated with tunicamycin suppressed the expression of PYCR1 protein, while it preserved the enhanced expression of the mutant BiP. The expression of CHOP, a cell

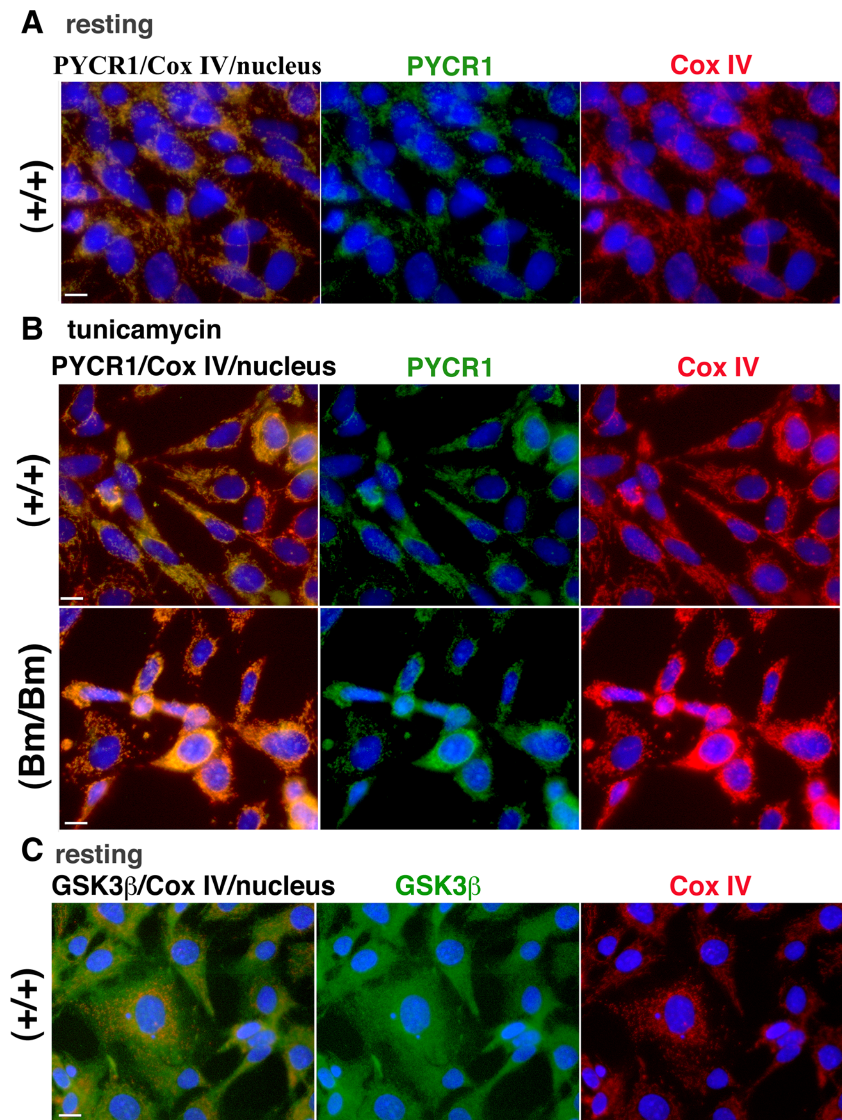

Fig. 3 Pyrroline-5-carboxylate reductase 1 (*PYCR1*) is localized to the mitochondrion in murine embryonic fibroblasts (MEFs). **a** MEFs from wild-type (+/+) embryos were fixed and stained with an anti-pyrroline-5-carboxylate reductase 1 (*PYCR1*) antiserum and an anti-cytochrome oxidase IV (*CoxIV*) mouse mAb. Nuclei were stained with Hoechst 33,342. **b** MEFs from wild-type (+/+) and homozygous BiP mutant (*Bm/Bm*) embryos were treated with tunicamycin (5 µg/ml, 12 h). They were fixed and stained with an anti-PYCR1 antiserum and an anti-cytochrome oxidase IV mouse mAb. Nuclei were stained with Hoechst 33,342. **c** MEFs from wild-type (+/+) embryos were fixed and stained with an anti-GSK3β rabbit antiserum and an anti-cytochrome oxidase IV mouse mAb. Nuclei were stained with Hoechst 33,342. *Scale bars* represent 20 µm (**a**, **b**, and **c**)

death related transcriptional factor during the UPR, was enhanced by the knockdown (Fig. 8). Thus, the upregulation of PYCR1 by the UPR was supposed to be cytoprotective.

In order to evaluate the synthesis of PYCR1 in vivo, we examined the expression of PYCR1 in the wild-type and mutant BiP brain. We found a small amount of PYCR1 expressed in adult wild-type and heterozygous mutant brains. The expression of PYCR1 seemed to be enhanced in the heterozygous mutant BiP brain with aging (Fig. 9a). The homozygous mutant embryonic brain expressed a further enhanced amount of PYCR1 as well as GRP94 and CHOP (Fig. 9b). The effect of the deletion of the KDEL sequence from BiP seems to be more significant in multicellular organisms rather than culture cells like MEFs. Since the homozygous mutant embryonic brain suffered from ER stress because of the absence of the wild-type BiP, sublethal ER stress induced the synthesis of a mitochondrial protein, PYCR1 in vivo.

Discussion

We found that sublethal ER stress caused by the BiP mutation enhanced the synthesis of a mitochondrial protein, PYCR1 in the mutant BiP embryonic brain in vivo. The enhanced synthesis of PYCR1 was also observed in MEFs upon sublethal ER stress with a tunicamycin treatment.

BiP is one of the most abundant ER chaperones and plays a central role in ER function, assisting in protein translocation, folding, degradation, and regulation of the UPR (Hendershot 2004). BiP is retained in the ER by interacting with other ER proteins and the ER matrix. When misfolded proteins accumulate in the ER, BiP dissociates from certain ER membrane proteins such as IRE1, PERK, and ATF6. This dissociation activates those kinases and initiates the UPR (Bertolotti et al. 2000), which causes an increased expression of X-box-binding protein-1 (XBP-1) and activating transcription factor

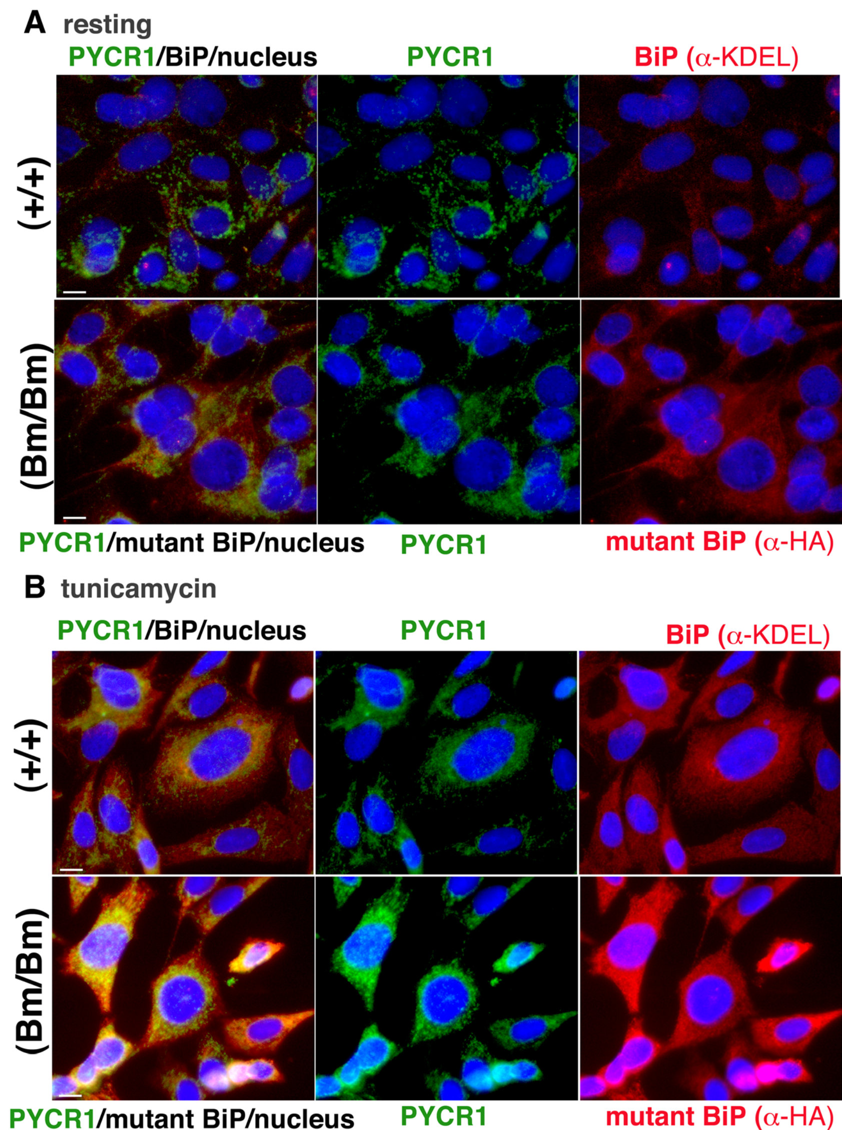

Fig. 4 Pyrroline-5-carboxylate reductase 1 (PYCR1) is localized to the mitochondrion in murine embryonic fibroblasts (MEFs). **a** MEFs from wild-type (+/+) and homozygous BiP mutant (*Bm/Bm*) embryos were fixed and stained with an anti-PYCR1 antiserum and an anti-KDEL mouse mAb for BiP or an anti-HA mouse mAb for mutant BiP. Nuclei were stained with Hoechst 33,342. *Scale bars* represent 20 μm. **b** MEFs treated with tunicamycin (5 μg/ml, 12 h) were stained in the same way

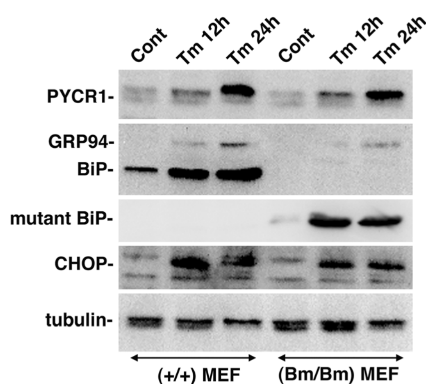

Fig. 5 The expression of pyrroline-5-carboxylate reductase 1 (PYCR1) was enhanced in both types of murine embryonic fibroblasts (*MEFs*) by ER stress with a tunicamycin treatment. Wild-type MEFs (+/+) and mutant BiP MEFs (*Bm/Bm*) were treated with tunicamycin (5 μg/ml for 12 and 24 h). Cells were subjected to a Western blot analysis with an anti-PYCR1 antiserum, an anti-KDEL mouse mAb for BiP and GRP94, an anti-HA mouse mAb for mutant BiP, an anti-CHOP rabbit antiserum, and an anti-tubulin mouse mAb

4 (ATF4) (Schroder and Kaufman 2005). When secreted from the ER in concert with misfolded proteins (Hammond and Helenius 1994; Yamamoto et al. 2001), the carboxyl-terminal KDEL sequence of BiP is recognized and retrieved by the KDEL receptor from the post-ER compartments to the ER (Lewis and Pelham 1990; Munro and Pelham 1987; Shen et al. 2002).

The complete depletion of BiP has lethal effects on murine embryonic cells (Luo et al. 2006). Deletion of the retrieval sequence in a cell is not lethal and is compensated by the UPR like in yeast (Beh and Rose 1995) and in MEFs (Mimura et al. 2007), while the homozygous mutant BiP mice whose retrieval sequence was deleted died within several hours after birth (Mimura et al. 2007). Although the UPR is induced, protein folding of certain essential proteins such as pulmonary surfactant protein C and reelin relies on the wild-type BiP in mice (Mimura et al. 2007; Mimura et al. 2008).

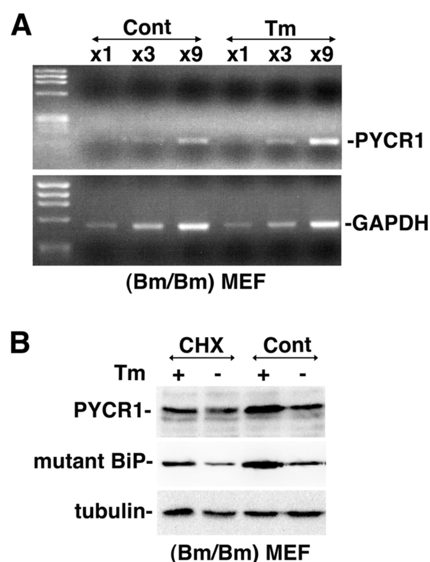

Fig. 6 Transcription of pyrroline-5-carboxylate reductase 1 (*PYCR1*) mRNA is up-regulated upon ER stress. **a** The mutant BiP MEFs (*Bm/Bm*) were treated with tunicamycin (Tm, 5 μg/ml for 24 h). cDNAs were synthesized based on mRNAs from the cells. The amount of PYCR1 mRNA in the mutant BiP MEFs was evaluated by semi-quantitative RT-PCR. Serial amounts of cDNAs were standardized by the expression of GAPDH. **b** Inhibition of protein translation during ER stress suppressed the expression of PYCR1 protein. The mutant BiP MEFs (*Bm/Bm*) were treated with tunicamycin (5 μg/ml for 24 h) and cycloheximide (CHX, 1 μg/ml for 24 h). Cells were subjected to a Western blot analysis with an anti-PYCR1 antiserum, an anti-HA mouse mAb for mutant BiP, and an anti-tubulin mouse mAb

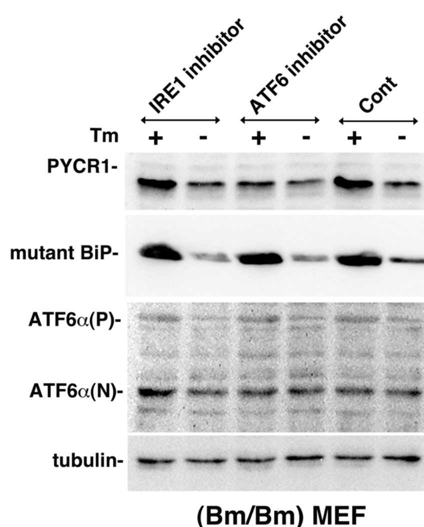

Fig. 7 The expression of pyrroline-5-carboxylate reductase 1 (*PYCR1*) was reduced in the mutant BiP murine embryonic fibroblasts (*MEFs*) by an ATF6 inhibitor under ER stress. Mutant BiP MEFs (*Bm/Bm*) were treated with tunicamycin (5 μg/ml for 24 h) and an IRE1 inhibitor (0.2 mM for 24 h) or an ATF6 inhibitor (60 μM for 24 h). Cells were subjected to a Western blot analysis with an anti-PYCR1 rabbit antiserum, an anti-HA mouse mAb for mutant BiP, an anti-tubulin mouse mAb, and an anti-ATF6α mouse mAb that detected both endogenous precursor ATF6α, ATF6α(P), and its cleaved product, ATF6α(N)

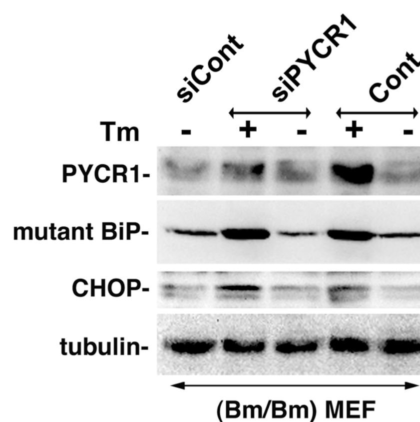

Fig. 8 Knockdown of PYCR1 enhanced the expression of CHOP in the mutant BiP MEFs during the UPR. The mutant BiP murine embryonic fibroblasts (*MEFs*) treated with siRNA for PYCR1 or control were incubated with tunicamycin (5 μg/ml) for 12 h at 37 °C. The cells were collected and subjected to a Western blot analysis with an anti-PYCR1 rabbit antiserum, an anti-HA mouse mAb for mutant BiP, an anti-CHOP rabbit antiserum, and an anti-tubulin mouse mAb

Therefore, we evaluated the effect of sublethal ER stress because of the BiP mutation by analyzing the profile of protein expressions in the homozygous mutant BiP brain. We found that the synthesis of a mitochondrial protein, PYCR1 was enhanced in the brains of homozygous mutant BiP mice. RT-PCR analysis revealed that the transcription of PYCR1 during ER stress was upregulated.

A previous study reported that the expression of PYCR1 mRNA was enhanced in transgenic mice with an activated form of ATF6 (Belmont et al. 2008). In fact, we found an ATF6 inhibitor, AEBSF, reduced the expression of PYCR1 under ER stress condition. AEBSF is a serine protease

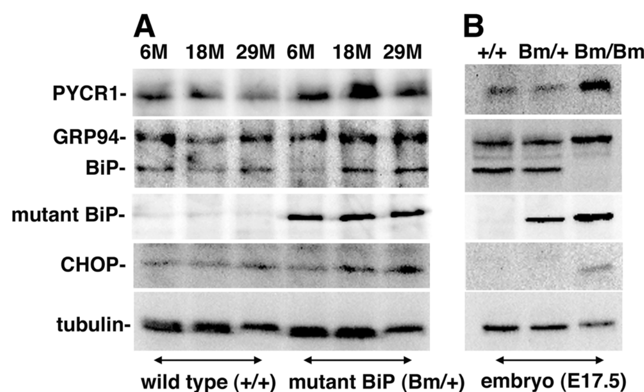

Fig. 9 The expression of pyrroline-5-carboxylate reductase 1 (*PYCR1*) was enhanced in homozygous mutant BiP mice. **a** Heterozygous mutant BiP mice (*Bm/+*) and litter mate wild-type mice (+/+, 6, 18, and 29 months old) were anesthetized by pentobarbital, and the brains were removed. **b** A pregnant mouse was anesthetized by pentobarbital. The brains from the litter mate embryos (homozygous; Bm/Bm, heterozygous; Bm/+ mutant BiP embryos and the wild-type embryo; +/+) were removed. They were subjected to a Western blot analysis with an anti-PYCR1 rabbit antiserum, an anti-KDEL mouse mAb for BiP and GRP94, an anti-HA mouse mAb for mutant BiP, an anti-CHOP rabbit antiserum, and an anti-tubulin mouse mAb

inhibitor that prevents ER stress-induced cleavage of ATF6, resulting in the inhibition of transcriptional induction of ATF6-target genes (Okada et al. 2003). Since AEBSF may not be specific for the processing of ATF6, other pathways may also be possible. The human PYCR1 gene has an AFT6-binding motif as well as a XBP1-binding motif (http://www.sabiosciences.com/chipqpcrsearch.). Another study reported that siRNA for ATF4 reduced the expression of PYCR1 mRNA in mouse L cells (Adams 2007). Thus, the transcription of PYCR1 mRNA is induced by the UPR, possibly through multiple pathways. Earlier work showed that the UPR affected multiple ER and secretory pathway functions rather than regulating only ER-resident chaperones using DNA microarrays (Travers et al. 2000). In this study, we found that the synthesis of PYCR1 protein, a mitochondrial protein, was indeed enhanced by sublethal ER stress because of the BiP mutation in mice and a tunicamycin treatment in culture cells.

PYCR1 is an enzyme that catalyzes the NAD(P)H-dependent conversion of pyrroline-5-carboxylate to proline and localizes to the mitochondrion. The PYCR1 gene is coded on chromosome 11 in mice and chromosome 17 in humans (Dougherty et al. 1992). Besides proline biosynthesis, PYCR1 colocalized with DJ-1 in the mitochondria, and both proteins were suggested to be involved in the regulation of the mitochondrial membrane potential and in the protection of cells against oxidative stress (Yasuda et al. 2013). Mutations in the human PYCR1 gene lead to autosomal recessive cutis laxa (ARCL) that is a group of syndromal disorders. ARCL is often associated with a progeroid appearance, lax and wrinkled skin, osteopenia, and mental retardation (Guernsey et al. 2009). Reversade et al. reported that altered mitochondrial morphology, membrane potential and increased apoptosis rate upon oxidative stress were evident in fibroblasts derived from affected human individuals with mutations in the gene PYCR1 (Reversade et al. 2009). They also described that knockdown of the orthologous genes in Xenopus and zebrafish led to epidermal hypoplasia and blistering that was accompanied by a massive increase of apoptosis (Reversade et al. 2009). Thus, PYCR1 proteins seem to be cytoprotective and anti-aging.

Recent studies have been accumulating evidences for interrelationships among intracellular organelles, such as ER and mitochondria (de Brito and Scorrano 2010; Vannuvel et al. 2013). ER stress beyond the capacity of quality control activates cell death pathways that are either dependent or independent on mitochondria (Tabas and Ron 2011). The activation of IRE1 by a prolonged UPR leads to the phosphorylation of JNK, which can activate the p53 transcription factor (Urano et al. 2000). P53 induces the expression of Bim and the formation of the BAX/BAK pore on the outer mitochondrial membrane (Han et al. 2010). Upon ER stress, calcium is released from the ER through inositol phosphate 3 receptors and taken by a mitochondrial calcium uniporter (Patron et al. 2013). An increase of the calcium concentration in the mitochondrial matrix results in the release of cytochrome *c* from the BAX/BAK pore of the mitochondria and the formation of the apoptosome in the cytosol, inducing activation of the caspase cascade and apoptosis (Mathai et al. 2005; Vannuvel et al. 2013).

The heterozygous mutant BiP mice grew up to be apparently normal adults. However, vulnerability to ER stress could result in chronic organ failure. Indeed, some of the mice displayed motor disabilities and nephropathy as they aged (Jin et al. 2014; Kimura et al. 2008). We found a degeneration of some motoneurons in the spinal cord accompanied by the accumulation of ubiquitinated proteins. Impairment of the PYCR1 function is a major cause of ARCL, evoking progeroid features (Guernsey et al. 2009). The impaired mitochondrial function leads to developmental defects through increased apoptosis (Reversade et al. 2009). Our finding that an impaired BiP function induced the synthesis of PYCR1 protein in vivo suggests that the interaction of ER chaperone and a mitochondrial protein plays an important role in aging as well as under stress conditions.

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Authors' contributions TA conceived and coordinated the study and wrote the article. HJ and TA designed, performed, and analyzed experiments. MK performed experiments. KS analyzed experiments and provided assistance for the preparation of paper. All authors reviewed the results and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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