FKBP8 protects the heart from hemodynamic stress by preventing the accumulation of misfolded proteins and endoplasmic reticulum-associated apoptosis in mice

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**ABSTRACT**

Protein quality control in cardiomyocytes is crucial to maintain cellular homeostasis. The accumulation of damaged organelles, such as mitochondria and misfolded proteins in the heart is associated with heart failure. During the process to identify novel mitochondria-specific autophagy (mitophagy) receptors, we found FK506-binding protein 8 (FKBP8), also known as FKBP38, shares similar structural characteristics with a yeast mitophagy receptor, autophagy-related 32 protein. However, knockdown of FKBP8 had no effect on mitophagy in HEK293 cells or H9c2 myocytes. Since the role of FKBP8 in the heart has not been fully elucidated, the aim of this study is to determine the functional role of FKBP8 in the heart. Cardiac-specific FKBP8-deficient (Fkbp8\textsuperscript{−/−}) mice were generated. Fkbp8\textsuperscript{−/−} mice showed no cardiac phenotypes under baseline conditions. The Fkbp8\textsuperscript{−/−} and control wild type littermates (Fkbp8\textsuperscript{+/+}) mice were subjected to pressure overload by means of transverse aortic constriction (TAC). Fkbp8\textsuperscript{−/−} mice showed left ventricular dysfunction and chamber dilatation with lung congestion 1 week after TAC. The number of apoptotic cardiomyocytes was dramatically elevated in TAC-operated Fkbp8\textsuperscript{−/−} hearts, accompanied with an increase in protein levels of cleaved caspase-12 and endoplasmic reticulum (ER) stress markers. Caspase-12 inhibition resulted in the attenuation of hydrogen peroxide-induced apoptotic cell death in FKBP8 knockdown H9c2 myocytes. Immunocytochemical and immunoprecipitation analyses indicate that FKBP8 is localized to the ER and mitochondria in the isolated cardiomyocytes, interacting with heat shock protein 90. Furthermore, there was accumulation of misfolded protein aggregates in FKBP8 knockdown H9c2 myocytes and electron dense deposits in perinuclear region in TAC-operated Fkbp8\textsuperscript{−/−} hearts. The data suggest that FKBP8 plays a protective role against hemodynamic stress in the heart mediated via inhibition of the accumulation of misfolded proteins and ER-associated apoptosis.

**1. Introduction**

Heart failure is one of the most common diseases affecting people worldwide and is associated with an increased risk of morbidity and mortality [1]. The accumulation of damaged proteins and organelles in failing hearts may be involved in the pathogenesis of heart disease [2]. As cardiomyocytes are terminally differentiated and long-lived cells, it is crucial to control the quality of proteins and organelles, such as mitochondria, under stress [3]. Chaperone/co-chaperone systems are engaged in protein folding and refolding, failure of which results in cellular toxicity [4]. The accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (ER) lumen, known as ER stress, activates unfolded protein response to maintain ER homeostasis by increasing ER-resident chaperones, inhibiting protein translation and...
accelerating the degradation of unfolded proteins [5].

The ubiquitin-proteasome system and autophagy lysosomal pathway are important for the degradation of intracellular components

The ubiquitin-proteasome system is involved in degrading misfolded proteins via proteolysis through an enzymatic cascade of ubiquitination, while autophagy is responsible for the bulk turnover of long-lived proteins and organelles. Autophagy includes not only non-selective autophagy, but selective autophagy, which targets specific proteins or organelles. Selective autophagy targeting mitochondria, mitophagy, is believed to be vital to remove damaged mitochondria. In yeast, autophagy-related (Atg) 32 protein is an essential mitophagy receptor, comprising a single transmembrane domain spanning the outer mitochondrial membrane and a WXXI motif, which binds to Atg8 [7]. We hypothesized that a mammalian mitophagy receptor would share the following molecular features with Atg32: mitochondrial localization, WXX/I motifs, and single membrane-spanning topology. The public protein database was screened for novel mammalian Atg32 functional homologue using the molecular profile of Atg32 as a search tool, identifying Bcl-2-like protein 13 (BCL2L13) that mediates mitophagy and mitochondrial fragmentation [8]. In addition to BCL2L13, FKS06-binding protein 8 (FKBP8), also known as FKBP38, satisfied the screening criteria.

FKBP8 is a member of the FKS06-binding protein family, which has a conserved peptidyl prolyl cis/trans-isomerase (PPIase) domain [9]. PPIase activity is important for both de novo folding of nascent polypeptide chains and the regulation of activities of mature client proteins. FKBP8 is membrane-anchored via the transmembrane domain and distributed predominantly in mitochondria [10]. FKBP8 mediates multiple functions such as protein folding and trafficking as a co-chaperone of heat shock proteins [11,12], apoptosis [10,13,14], cell size regulation [15] and the mammalian target of rapamycin (mTOR) signaling [16]. Conventional FKBP8-deficient mice die at embryonic day 13.5 or shortly after birth due to prominent malformation of the nervous system including defect in neural tube closure [17,18]. However, the role of FKBP8 in the heart has not been fully elucidated.

The aim of the present study was to determine the functional role of FKBP8 in the heart. Cardiac-specific FKBP8-deficient mice were generated for this purpose and provided the first evidence that FKBP8 plays a protective role against pressure overload in the heart.

2. Methods

Detailed methods are provided in the Online Supplement.

2.1. Generation of cardiac-specific FKBP8-deficient mice

We generated mice with the Fkbp8lox allele and crossed them with mice expressing α-myosin heavy chain promoter-driven Cre recombinase transgenic mice (αMHC-Cre) to obtain cardiac-specific FKBP8-deficient mice [19]. All procedures were carried out in accordance with the King’s College London Ethical Review Process Committee and UK Home Office (Project License No. PPL70/7260 and 70/8889).

2.2. Transverse aortic constriction (TAC) and echocardiography

The 8–11 weeks old mice were subjected to TAC or sham surgeries as previously reported [20]. Echocardiography was conducted with a Vevo 2100 system (Visual Sonics, Toronto, Canada) on conscious mice [21].

2.3. Western blot analysis and mitochondrial fractioning

Western blot analysis from frozen left ventricular tissues or cultured cells was performed as previously described [21]. Mitochondrial fractions were isolated from mouse hearts by differential centrifugation methods [22].

2.4. Histological analysis

O.C.T.-embedded samples were stained with hematoxylin-eosin, Masson's trichrome (Masson's Trichrome Stain Kit, Polysciences, Warrington, PA), wheat germ agglutinin (Alexa Fluor 488 conjugated, Thermo Fisher Scientific, Waltham, MA), or immunohistochemical staining with S100A4 (FSP1) antibody (ab41532, Abcam, Cambridge, UK) followed by avidin peroxidase (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA), DAB peroxidase substrate kit (Vector Laboratories) and counterstaining with hematoxylin. Fibrosis fraction and cross-sectional areas of cardiomyocytes were measured using the NIH ImageJ software (National Institutes of Health, Bethesda, MD) [22].

2.5. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the left ventricle using the Trizol reagent (Thermo Fisher Scientific). RT-qPCR was performed to determine mRNA levels for Nppa, Npb, Myh7, Col3a1, Col1a2. All data were normalized to Actb and expressed as a fold increase of the control group [21]. Primer sequences were described in the Online Supplement.

2.6. Transmission electron microscopy

The heart was perfused with ice cold 0.1 mmol/L PIPES buffer containing 2.5% polyvinylpyrrolidone and 0.1% sodium sitrite, and then fixed with 0.1 mmol/L PIPES buffer containing 2% glutaraldehyde, 2% paraformaldehyde, 0.1% sodium itrite and 2.5% polyvinylpyrrolidone. H9c2 cells were fixed with 2.5% glutaraldehyde in 0.1 mmol/L cacodylate buffer. Samples were processed according to our standard procedures [22].

2.7. Mitochondrial enzyme activities

The mitochondrial fraction was freshly prepared from mouse hearts. Mitochondrial electron transport chain complex activities of NADH cytochrome-c oxidoreductase (complex I + III) and succinate cytochrome-c oxidoreductase (complex II + III) were evaluated using spectrophotometric methods [22]. The data were expressed as a relative ratio to the control group.

2.8. Terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) staining

O.C.T.-embedded heart sections were stained with TUNEL kit (Takara Bio, Otsu, Japan) and anti-actin (α-sarcomeric) antibody (A2172, Sigma-Aldrich, St. Louis, MO) followed by the secondary antibody Texas Red Anti-Mouse IgM (Vector Laboratories). For H9c2 cells, the cells were fixed in 4% paraformaldehyde, stained with the TUNEL kit. Samples were mounted with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). The number of TUNEL-positive nuclei and total nuclei was counted, and expressed as the number of TUNEL-positive cardiomyocytes per 10^4 nuclei for the heart tissue and as the percentage of TUNEL-positive nuclei to total nuclei for H9c2 cells.

2.9. Cell culture and transfection with small interfering RNA (siRNA) and plasmid DNA

H9c2 rat embryonic cardiac myoblasts and HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 μg/mL of streptomycin and 100 IU/mL of penicillin at 37 °C in the presence of 5% CO₂. For siRNA transfection in H9c2 cells, cells were transfected with scrambled negative control siRNA (4390843,
Thermo Fisher Scientific) orFKBP8-specific siRNA (4390771, s66102, Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to manufacturer’s instructions. For plasmid DNA transfection, HEK293 cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer’s protocols.

2.10. Cell viability assay

The numbers of viable cells were analyzed using a Cell-Titer Blue assay (Promega, Fitchburg, WI). The caspase-12 specific inhibitor, Z-ATAD-FMK (BioVision, Milpitas, CA), was incubated prior to hydrogen peroxide (Sigma-Aldrich) stimulation. The data were expressed as a relative ratio to the control.

2.11. Isolation of mouse adult cardiomyocytes and rat neonatal cardiomyocytes and immunofluorescence

Mouse adult cardiomyocytes were isolated using a Langendorff system as previously reported [20]. Rat neonatal cardiomyocytes were isolated from hearts of 1 to 2 day old Sprague-Dawley rats [20]. Immunofluorescence was performed as described in the Online Supplement.

2.12. Construction of DNA plasmid

Constructs of mouse Fkbp8 (NM_001111066.1) and heat shock protein 90 alpha family class A member 1 (Hsp90aa1) (NM_010480.5) were obtained by conventional restriction enzyme-based cloning. Detailed methods were described in the Online Supplement.

2.13. Immunoprecipitation

Samples were prepared from HEK293 cells transfected with plasmid DNA or mouse heart tissue. Detailed methods were described in the Online Supplement.

2.14. Detection for protein aggregates

Protein aggregates were detected with Proteostat Aggresome Detection Kit (Enzo Life Sciences, Farmingdale, NY) according to manufacturer’s instruction [23]. H9c2 cells were seeded onto coverslips and transfected with siRNA, and then cells were fixed with 4% paraformaldehyde, permeabilized by Triton X-100, labeled with Proteostat aggresome dye, and mounted with ProLong Gold Antifade Reagent with DAPI. For co-staining with Proteostat dye, cells were incubated with Proteostat dye, and mounted by ProLong Gold Antifade Reagent with DAPI. Samples were prepared from HEK293 cells transfected with plasmid DNA or mouse heart tissue. Detailed methods were described in the Online Supplement.

2.15. Statistical analysis

All data are expressed as the mean ± SEM. Student’s t-test was used for the comparison of paired data. One way analysis of variance followed by the Bonferroni’s post hoc test was applied for multiple comparisons. All data were analyzed with IBM SPSS Statistics 22.0 (IBM, Armonk, NY). A probability value < 0.05 was considered statistically significant.

3. Results

3.1. FKBP8 is not involved in mitochondrial damage-induced mitophagy

Mouse Fkbp8 encodes 403 amino acids containing a C-terminal single transmembrane domain and sequence alignment revealed two WXXL/I motifs at positions 83–86 and 379–382, which are consensus sequences for the binding sites of microtubule associated protein 1 light chain 3 (LC3), a mammalian homologue of Atg8 (Supplementary Fig. 1A) [24]. Firstly, we tested whether FKBP8 interacts with LC3B mediated through the WXXL/I motifs. A yeast two-hybrid assay using Gal4-fused LC3B and activation domain-fused FKBP8 showed that the cells expressing LC3B and FKBP8 grew on selective plates (Supplementary Fig. 1B). The FKBP8 mutant, which contains W83A I86A W379A L382A amino acid substitution in the WXXL/I motifs, also could grow on the plates. Thus, the interaction between FKBP8 and LC3B was not mediated via the WXXL/I motifs, but seems to be rather non-specific. To estimate the role of FKBP8 in mitophagy, FKBP8 was knocked down in a stable cell line of HEK293 expressing mitochondrial targeted mKeima [8], a coral-derived acid-stable lysosomal proteases-resistant fluorescent protein. Knockdown of endogenous FKBP8 was performed using siRNA (Supplementary Fig. 1C). The knockdown cells were incubated with a mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which induces mitophagy, and stained with Lysotracker Green before microscopic analysis (Supplementary Fig. 1D). The high-ratio mKeima dots were colocalized with Lysotracker-positive dots, confirming lysosomal localization. Knockdown of FKBP8 did not change the number of high-ratio mKeima and Lysotracker double positive dots, suggesting that FKBP8 is not involved in CCCP-induced mitophagy (Supplementary Fig. 1E). Furthermore, GFP-LC3 was transfected in FKBP8 knockdown H9c2 myocytes [25]. The number of mitochondria positive for GFP-LC3-labeled autophagic structures was significantly elevated after CCCP stimulation, but there was no difference between control and FKBP8 knockdown H9c2 myocytes (Supplementary Fig. 1F).

To clarify the role of FKBP8 during cardiac remodeling, the change in FKBP8 expression levels in wild type C57BL/6J mouse hearts after pressure overload was estimated. In our model, mice show cardiac hypertrophy without cardiac dysfunction 1 week after TAC, while they exhibit heart failure phenotypes 4 weeks after the operation. The protein level of FKBP8 in the heart was upregulated at both 1 and 4 weeks after TAC compared to the corresponding sham-operated mice (Supplementary Fig. 2), indicating that FKBP8 may play a pathophysiological role during pressure overload-induced cardiac remodeling.

3.2. Generation and characterization of cardiac-specific FKBP8-deficient mice

Cardiac-specific FKBP8-deficient mice were generated to establish the in vivo role of FKBP8 in the heart. We designed a gene targeting strategy to conditionally inactivate the Fkbp8 gene by insertingloxP sites in intron 2 and 6 (Fig. 1A). The neomycin resistance gene was inserted between exon 6 and the downstreamloxP site. The diphtheria toxin A gene was positioned at the 3’ end of the targeting construct for negative selection. Homologous recombinants were identified by PCR and Southern blot analysis (Fig. 1B). The ES cells with Fkbp8-flxed allele were injected into blastocysts to obtain Fkbp8flx/flx/mice. The homozygous floxed Fkbp8 mice (Fkbp8flx/flx) appeared normal and were externally indistinguishable from littermates of other genotypes. Fkbp8flx/flx/mice were crossed withαHMC-Cre mice [19] to generate Fkbp8flx/flx,αHMC-Cre+(Fkbp8flx/flx) mice. Fkbp8flx/flx,αHMC-Cre− littermates were used as controls (Fkbp8flx/flx). The Fkbp8+/+/ and Fkbp8−/−/−mice were born normally at the expected Mendelian ratio (58 and 55 mice, respectively), and they grew to adulthood and were fertile. Then, the efficiency of Fkbp8 ablation was determined in the heart by immunoblot analysis. The protein level of FKBP8 significantly decreased in Fkbp8−/−hearts by 88% compared to Fkbp8+/+/mice (Fig. 1C). Echocardiographic analysis revealed that ablation of Fkbp8 in cardiomyocytes had no effect on cardiac morphology and function.
under baseline conditions at 8–10 weeks old (Supplementary Table 1). No differences in any physiological parameters were observed in Fkbp8+/− mice compared to Fkbp8+/+ mice (Supplementary Table 1), indicating that Fkbp8+/− mice showed normal global cardiac structure and function.

3.3. FKB8-deficient mice developed heart failure in response to pressure overload

To examine the role of FKB8 in the development of cardiac remodeling, mice were subjected to pressure overload by means of TAC [26]. Echocardiographic analysis was performed 1 week after TAC (Fig. 2A and B). The diastolic interventricular septum wall thickness (IVSD) and left ventricular (LV) posterior wall thickness (LVPWd) were elevated in TAC-operated Fkbp8+/− and Fkbp8+/− mice compared to the corresponding sham-operated mice. While IVSd was not different between TAC-operated Fkbp8+/− and Fkbp8−/− mice, LVPWd in TAC-operated Fkbp8−/− mice was significantly reduced compared to TAC-operated Fkbp8+/− mice. The calculated LV mass was elevated by TAC, but not different between TAC-operated Fkbp8−/− and Fkbp8+/− mice. The end-diastolic and end-systolic LV internal dimensions were significantly elevated and fractional shortening, an index of contractility, was reduced in TAC-operated Fkbp8−/− mice compared to both sham-operated Fkbp8+/− and TAC-operated Fkbp8+/− mice. Although TAC increased heart weight-to-tibia length ratio in both Fkbp8+/− and Fkbp8−/− mice, there was no significant difference between the two groups (Fig. 2C). Lung weight-to-tibia length ratio, an index of lung congestion, was significantly elevated in TAC-operated Fkbp8−/− mice compared to both sham-operated Fkbp8+/− and TAC-operated Fkbp8+/− mice. These data suggest that Fkbp8−/− mice developed congestive heart failure in response to pressure overload.

3.4. TAC-operated FKB8-deficient mice exhibited myocardial remodeling

Histological examination showed that FKBP8 deficiency significantly elevated heart weight-to-tibia length ratio in TAC-operated Fkbp8−/− hearts 1 week after TAC (Fig. 3A). Masson’s trichrome staining revealed interstitial fibrosis in both TAC-operated Fkbp8+/− and Fkbp8−/− hearts, but the extent of fibrosis in Fkbp8−/− mice was greater than in Fkbp8+/− mice (Fig. 3A, B and Supplementary Fig. 3). Immunohistological examination using anti-fibroblast-specific protein-1 antibody indicated that the infiltrating cells in TAC-operated Fkbp8−/− hearts were mainly fibroblasts (Fig. 3A). Although wheat germ agglutinin staining showed TAC increased the cross-sectional area of cardiomyocytes in both Fkbp8+/− and Fkbp8−/− mice, there was no significant difference between the two groups (Fig. 3A and C). TAC-operated Fkbp8−/− mice displayed higher mRNA expression of Nppa, which is a biochemical marker for cardiac remodeling, than TAC-operated Fkbp8+/− and TAC-operated Fkbp8+/− mice (Fig. 3D). Although Nppb and Myh7 mRNA expression were elevated by pressure overload in both Fkbp8+/− and Fkbp8−/− mice, there were no significant differences between the two groups. The mRNA levels of Col1a2 and Col3a1, markers for fibrosis, were significantly higher in TAC-operated Fkbp8−/− mice than those in TAC-operated Fkbp8+/− mice. These data indicate that deficiency of FKB8 exacerbated TAC-induced cardiac remodeling including cardiac dysfunction, chamber dilatation and fibrosis, but FKB8 was not involved in TAC-induced cardiac hypertrophy.

3.5. Deficiency of FKB8 had no effect on mitochondrial morphology, enzymatic function, mitophagy or mTOR signaling

Since FKB8 is reported to be expressed in outer mitochondrial membrane [10], mitochondrial morphology and function were examined to explore the mechanisms underlying the cardiac phenotypes observed in TAC-operated Fkbp8+/− mice. Ultrastructural analysis demonstrated that sarcomere structures were preserved in Fkbp8+/− hearts 1 week after TAC and there was no apparent difference in mitochondrial morphology and intra-mitochondrial structures among groups (Fig. 4A). Mitochondrial functions estimated by the enzymatic activity of complex I + III and II + III were not different among groups (Fig. 4B). Likewise, the protein expression levels of mitochondrial proteins such as succinate dehydrogenase complex flavoprotein subunit A (SDHA), cytochrome c oxidase subunit IV (COX IV), and voltage-dependent anion channel (VDAC) were not different among groups (Fig. 4C). For the assessment of mitophagy in the heart, mitochondrial DNA content which can be degraded by mitophagy [27] and the level of LC3B-II in mitochondrial fraction which can reflect mitochondrial autophagosome formation [28] were examined, and there were no differences among groups (Supplementary Fig. 4A and 4B). Although it has been recently reported that FKB8 recruits lipitated LC3A to mitochondria [29], the level of LC3A-II in mitochondrial fraction was not different among groups (Supplementary Fig. 4C). Thus, these results
suggest that mitochondrial morphological change, function, and mitophagy were not associated with cardiac dysfunction observed in TAC-operated Fkbp8−/− mice. It has been reported that FKBP8 is an endogenous inhibitor of mTOR [16]. Although phosphorylation levels of both S6 and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), downstream signaling molecules of mTORC1, were significantly elevated by TAC, there were no differences between TAC-operated Fkbp8+/+ and Fkbp8−/− hearts (Fig. 4D), indicating that mTOR signaling is not involved in cardiac phenotypes observed in Fkbp8−/− hearts after hemodynamic stress.

3.6. Cardiomyocyte apoptosis increased in FKB8-deficient hearts after TAC

Apoptotic cardiomyocyte death plays an important role in the development and progression of heart failure [30]. Cardiomyocyte apoptosis was evaluated in hearts 1 week after TAC by means of TUNEL assay and immunoblot analysis using anti-caspase antibodies. The numbers of TUNEL-positive cardiomyocytes in sham-operated Fkbp8−/− mice were similar (Fig. 5A). However, TAC-operated Fkbp8−/− mice exhibited more TUNEL-positive cells.
cardiomyocytes than TAC-operated Fkbp8+/− mice. The expression level of cleaved caspase-3 was significantly higher in TAC-operated Fkbp8−/− hearts than in TAC-operated Fkbp8+/− hearts (Fig. 5B). When the activation levels of the initiator caspases upstream of caspase-3 were evaluated, the expression of cleaved caspase-8 and caspase-9, which are involved in the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, respectively, was not detectable in all groups. The expression levels of cleaved caspase-12, which is specifically associated with ER stress-mediated apoptosis, were significantly elevated in TAC-operated Fkbp8−/− hearts compared to both sham-operated Fkbp8−/− and TAC-operated Fkbp8+/− hearts. Although the protein expression level of Bax or Bcl-2 in isolated mitochondrial fraction was significantly elevated in TAC-operated Fkbp8−/− hearts compared to sham-operated Fkbp8−/− hearts, there was no significant difference in Bax or Bcl-2 level between TAC-operated Fkbp8−/− and Fkbp8+/− hearts (Fig. 5C). The ratios of Bax to Bcl-2 protein were not different among the four groups. To support the involvement of ER stress-associated cell death in the genesis of cardiac dysfunction in Fkbp8−/− mice, the expression levels of ER stress markers such as glucose-regulated protein 94 kDa (GRP94), glucose-regulated protein 78 kDa (GRP78) and protein disulfide-isomerase (PDI) were assessed. These ER stress markers were significantly elevated by TAC in both Fkbp8+/− and Fkbp8−/− mice, but these protein levels in TAC-operated Fkbp8−/− hearts were higher than in TAC-operated Fkbp8+/− hearts (Fig. 5D). Furthermore, phosphorylation level of protein kinase R–like endoplasmic reticulum kinase (PERK) and protein expression level of activating transcription factor 4 (ATF4) were significantly upregulated in TAC-operated Fkbp8−/− hearts (Supplementary Fig. 5).

3.7. Caspase-12 inhibition effectively attenuated H2O2-induced cardiomyocyte apoptosis in Fkbp8-deficient H9c2 cells

ER stress and oxidative stress are closely linked and reactive oxygen species (ROS) can cause protein misfolding in the ER, leading to ER stress [31]. Although pressure overload significantly promoted ROS generation in the heart assessed by heme oxygenase 1 (HO-1) expression, there was no difference between TAC-operated Fkbp8−/− and Fkbp8+/− hearts (Supplemental Fig. 6). To investigate the role of caspase-12 in Fkbp8-related apoptotic cell death, the effect of caspase-12 inhibition on hydrogen peroxide (H2O2)-induced cell death in Fkbp8 knockout H9c2 myocytes was examined. The Fkbp8 siRNA (siFkbp8) significantly decreased the endogenous protein levels of Fkbp8 compared to control siRNA (siCTRL) (Fig. 6A). We incubated Fkbp8 knockout cells with 50 μmol/L of H2O2 for 6 h to evaluate cell viability. There was no difference in cell viability between cells transfected with siCTRL and siFkbp8 without H2O2 stimulation (Fig. 6B). Although H2O2 did not induce cell death in siCTRL-transfected cells in our experimental conditions, the cell viability was significantly reduced in H2O2-treated Fkbp8 knockout myocytes, indicating that Fkbp8 is involved in H2O2-induced cell death. Z-ATAD-FMK, which is a caspase-12 inhibitor, improved cell viability in H2O2-treated Fkbp8 knockout H9c2 myocytes. H2O2 increased the number of TUNEL-positive nuclei in Fkbp8 knockout myocytes, while Z-ATAD-FMK treatment attenuated the increase in apoptotic cell number (Fig. 6C). Furthermore, the levels of cleaved caspase-3 and caspase-12 in Z-ATAD-FMK- and H2O2-treated Fkbp8 knockout cells were lower than in vehicle- and H2O2-treated Fkbp8 knockout cells (Fig. 6D). However, Z-ATAD-FMK had no effect on H2O2-induced upregulation of GRP94 and GRP78 in Fkbp8 knockout myocytes. These data indicate caspase-12 is...
involved in H2O2-induced apoptotic cell death in FKBP8 knockdown myocytes and exists downstream of ER stress.

3.8. FKBP8 was localized at the ER in the isolated cardiomyocytes and bound to heat shock protein 90 (HSP90)

We then examined subcellular localization of FKBP8 in the cardiomyocytes. Confocal microscopic analysis using anti-KDEL and anti-Tomm20 antibodies demonstrated that endogenous FKBP8 was localized to both ER and mitochondria in adult cardiomyocytes isolated from Fkbp8+/+ mouse heart (Fig. 7A and B). Triple labeling of endogenous FKBP8 and KDEL in combination with MitoTracker was performed in cultured rat neonatal cardiomyocytes. We focused on areas where the ER is enriched (Supplementary Fig. 7A) or tubular structure of mitochondria is evident (Supplementary Fig. 7B). The results provided further evidence of localization of FKBP8 in both ER and mitochondria. To confirm ER localization of FKBP8, rat neonatal cardiomyocytes were transfected with tdTomato-ER-3 containing KDEL sequence to target it to the ER. The expression of tdTomato showed a clear mesh pattern in the peripheral area of the cells, which is characteristic of ER structure, and was colocalized with endogenous FKBP8 staining (Fig. 7C).

To investigate how FKBP8 is involved in ER quality control, we examined whether FKBP8 interacted with chaperon proteins such as heat shock proteins. It has been reported that FKBP8 interacted with HSP90, which is a major molecular chaperone to assist protein folding [4,12,32]. The communoprecipitation assay demonstrated the interaction of FKBP8 with HSP90 in HEK293 cells in the absence or presence of Ca2+ (Fig. 7D). Using protein lysates from mouse hearts, endogenous FKBP8 was shown to interact with HSP90 (Fig. 7E). Taken together, these data suggest that FKBP8 might act as a co-chaperone coordinating with HSP90 at the ER.

3.9. Deficiency of FKBP8 resulted in the accumulation of misfolded proteins in cardiomyocytes

ER stress is the consequence of the accumulation of misfolded proteins. The aggregation of misfolded proteins was visualized using Proteostat aggresome detection dye, a molecular rotor fluorescent dye which specifically detects misfolded protein aggregates [23,33]. Knockdown of FKBP8 in H9c2 cells induced the accumulation of Proteostat-positive aggregates in the perinuclear region as well as in cultured rat neonatal cardiomyocytes (Fig. 8A and Supplementary Fig. 8A). Next, ultrastructure of misfolded protein aggregates was characterized. There were aggresome-like electron dense deposits with membranous contents and vacuoles in FKBP8 knockdown H9c2 cells (Fig. 8B). We examined the effect of H2O2 stimulation on misfolded protein aggregates. We observed the cells 1.5 h after H2O2 administration, when cell viability was maintained (Fig. 8C). H2O2 did not increase the percentage of Proteostat-positive cells in FKBP8 knockdown or control cells. The p62 facilitates degradation of ubiquitinated protein aggregates by autophagy [34]. We examined whether the Proteostat-positive structures in FKBP8 knockdown H9c2 cells were ubiquitinated and/or contained p62 using anti-ubiquitin or anti-p62...
antibody and found that the Proteostat-positive structures were not costained with ubiquitin nor p62 (Fig. 8D and E).

Since the ubiquitin-proteasome system and autophagy lysosomal pathway are important for the degradation of misfolded proteins [2], proteasome and autophagy activity were investigated in FKBP8 knockout H9c2 cells (Supplementary Fig. 8B and 8C). Knockdown of FKBP8 in H9c2 cells had no effect on proteasome activity nor the protein level of LC3B-II. Furthermore, Tat-Beclin 1, an autophagy inducible peptide [35], increased the level of LC3B-II (Supplementary Fig. 8D), but had no effect on the accumulation of Proteostat-positive structures or cellular viability in FKBP8 knockout H9c2 cells (Supplementary Fig. 8F and G).

Protein aggregates in pressure overloaded hearts are observed in the perinuclear area as electron dense deposits [36]. Ultrastructural analysis showed that TAC-operated Fkbp8−/− hearts displayed disorganization in the perinuclear region with high electron dense structures and membranous structures (Fig. 8F). Taken together, these findings suggest that deficiency of FKBP8 resulted in the accumulation of misfolded protein during hemodynamic stress, leading to cardiac dysfunction.

4. Discussion

The purpose of this study was to elucidate the role of FKBP8 in the heart and it was the first to identify the in vivo role of FKBP8 in the heart using a loss-of-function mouse model. We showed that cardiac-specific FKBP8-deficient mice had no effect on cardiac structure or function under basal conditions, indicating that FKBP8 is not essential for cardiac development or postnatal growth. It is possible that other molecular pathways could compensate the loss of FKBP8 function. In stressed hearts, FKBP8 has a cardio-protective role in response to pressure overload by preventing misfolded proteins and resultant ER-associated apoptosis.

Although Atg32 is essential for mitophagy in yeast, no mammalian homologue has been identified. We hypothesized that a mammalian mitophagy receptor would share certain molecular features with Atg32 and identified BCL2L13 and FKBP8 as possible candidates. BCL2L13 mediates mitochondrial fragmentation and mitophagy in HEK293 cells [8]. However, knockdown of FKBP8 had no effect on CCCP-induced mitophagy in HEK293 cells or H9c2 myocytes. Furthermore, the FKBP8 mutant containing amino acid substitutions in the WXXL/I motifs was able to bind LC3B. These data suggest that FKBP8 is not involved in mitophagy. Recently, Bhujabal et al. reported that FKBP8 is a novel mitophagy receptor which recruits LC3A and identified the FEVL motif.
located in the N-terminal site of FKBP8 as a binding site for LC3A [29]. They demonstrated that co-overexpression of FKBP8 and LC3A increased the percentage of the cells containing acidified mitochondria, while overexpression of FKBP8 alone did not activate mitophagy. They also showed FKBP8 recruited lipidated LC3A to mitochondria upon CCCP treatment when the LC3A was exogenously overexpressed in HeLa and HEK293 cells, but our results indicate that endogenous LC3A-II level in mitochondrial fraction was not altered in TAC-operated Fkbp8−/− hearts. Although we cannot fully explain this discrepancy regarding the role of FKBP8 in mitophagy, overexpression of FKBP8 with LC3A might not specifically induce mitophagy.

In this study, ablation of Fkbp8 induced dilated cardiomyopathy and massive cardiomyocyte apoptosis with the activation of caspase-12 and increase in ER stress markers in response to pressure overload. Thus, FKBP8 is indispensable for cardiac adaptation against hemodynamic stress. Caspase-12 is localized in the ER and involved in ER stress-induced apoptosis [5], ER stress results from the accumulation of excess proteins and misfolded proteins in the ER lumen. When the misfolded protein load exceeds the capacity, ER-initiated cell death is induced via the caspase-12 signaling pathway. There is debate concerning the subcellular localization of FKBP8, with some reports that FKBP8 is localized predominantly to the outer mitochondrial membrane through its C-terminal membrane anchor in HeLa cells [10], while others have shown that FKBP8 is localized to the ER in HEK293 cells and both ER and mitochondria in SH-SYSY cells [12,37]. Our results showed that FKBP8 is highly expressed in the ER as well as in mitochondria under basal conditions in cardiomyocytes. It has been reported that FKBP8, along with Bcl-2, translocates from mitochondria to the ER during CCCP-induced mitophagy in mouse embryonic fibroblasts [38]. Mitophagy is known to be transiently upregulated in response to pressure overload in mouse hearts [27]. We attempted to detect TAC-induced changes in subcellular distribution of FKBP8, but due to high background signal in heart sections using commercially available anti-FKBP8 antibodies, it was not possible to prove the translocation of FKBP8 from mitochondria to the ER upon pressure overload. However, TAC did not change mitochondrial protein levels in Fkbp8−/− hearts and the levels were not different between TAC-operated Fkbp8+/− and Fkbp8−/− hearts. In addition, mitochondrial Bcl-2 levels were similar between sham- and TAC-operated Fkbp8+/− hearts and those in TAC-operated Fkbp8−/− hearts were not different compared with TAC-operated Fkbp8+/− hearts. Although the extent of mitophagy in pressure overloaded hearts may not be sufficient to induce global degradation of mitochondrial protein content, translocation of FKBP8 with Bcl-2 from mitochondria to the ER during mitophagy was not evident in our study. The distribution of FKBP8 in the cell might be cell-type specific or depend on cellular conditions under stress.

It has been reported that FKBP8 is involved in apoptosis. FKBP8 binds to Bcl-2 and Bcl-xI, recruiting them to mitochondria and inhibiting mitochondria-initiated apoptosis in HeLa cells [10]. FKBP8 translocation to the ER prevents unwanted apoptosis during mitophagy induced by CCCP [38]. Conversely, in neuronal SH-SYSY cells, association between Bcl-2 and Ca2+/calmodulin/FKBP8 complex participates in the promotion of apoptosis [37]. The in vitro and in vivo studies presented here clearly showed the anti-apoptotic function of FKBP8 in cardiomyocytes. The function of FKBP8 in apoptosis is perhaps cell-type specific due to various Bcl-2 interaction partners and their expression pattern [37]. In this study, we detected an increase in protein levels of cleaved caspase-12, but not that of cleaved caspase-8 or 9 in TAC-operated Fkbp8−/− hearts. Furthermore, the Bax/Bcl-2 ratio in the mitochondrial fraction was not different between TAC-operated Fkbp8+/− hearts and those in TAC-operated Fkbp8−/− hearts.
and Fkbp8<sup>−/−</sup> hearts. In addition, the inhibition of caspase-12 attenuated H₂O₂-induced cell death in H9c2 cells. Taken together, these data indicate that FKBP8 is involved in caspase-12-mediated apoptosis, but not in the mitochondrial apoptotic pathway. Apoptotic death of cardiomyocytes plays a pivotal role in the progression of cardiac remodeling [30]. Considering the very high level of apoptosis in TAC-operated Fkbp8<sup>−/−</sup> hearts, the cardiomyopathic phenotypes observed in TAC-operated Fkbp8<sup>−/−</sup> mice would be due to the upregulation of caspase-12-mediated apoptosis.

Protein misfolding is linked to the pathogenesis of many diseases including heart failure and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease [3]. The protein aggregates, which are transported on the microtubules and accumulate in the perinuclear region, have been termed aggresomes [39]. Reports of ubiquitin localization to aggresomes are inconsistent. The p62 facilitates degradation of ubiquitinated protein aggregates by autophagy [34]. Desmin-related cardiomyopathy is a well-studied disease, where misfolded proteins are implicated in cardiac dysfunction [2]. The αB-crystallin (CryAB) is a small chaperone of desmin protein. The transgenic mice expressing mutant CryAB (R120G) in the heart displayed the accumulation of protein aggregates containing desmin and dilated cardiomyopathy [40]. Overexpression of CryAB (R120G) in rat neonatal cardiomyocytes resulted in ubiquitin-positive aggresome formation in the perinuclear region [41]. Activation of autophagy attenuated the development of cardiomyopathy in the CryAB (R120G) transgenic mice [42]. In wild type mice, pressure overload promotes development of ubiquitinated aggresome-like structures in the perinuclear region with upregulation of autophagy in the heart [36]. Thus, the continuous presence and chronic accumulation of misfolded proteins in cardiomyocytes can lead to the development of heart failure [2]. Intracellular protein aggregation is a proximal trigger of cardiomyocyte autophagy [31]. In this study, we observed the accumulation

![Fig. 8. Knockdown or knockout of FKBP8 results in the accumulation of misfolded protein in cardiomyocytes.](chart)

A, Detection of misfolded protein aggregates in H9c2 cells. Ninety-six h after transfection with siCTRL or siFKBP8, the cells were stained with Proteostat fluorescent dye (red) and DAPI (blue), and subjected to confocal microscopic analysis. Boxed areas at higher magnification (high) are shown in right panels. Scale bars are 100 μm and 10 μm, respectively. B, Electron micrographs of H9c2 cells transfected with siCTRL or siFKBP8. Boxed areas at higher magnification are shown in right panels. Scale bars are 10 μm and 2 μm, respectively. C, The effect of H₂O₂ on protein aggregates. H9c2 cells transfected with siCTRL or siFKBP8 were fixed 1.5 h after H₂O₂ stimulation and stained with Proteostat dye (red) and DAPI (blue). The percentage of Proteostat-positive cells to total nuclei is shown in the graph (n = 3). More than 50 cells were counted in each group. *p < 0.05 versus the corresponding siCTRL group. Scale bar, 50 μm. D, Detection of ubiquitinated protein and p62 in Proteostat-positive structures. Ninety-six h after transfection with siCTRL or siFKBP8, the cells were stained with Proteostat fluorescent dye (red), followed by anti-ubiquitin (green) or anti-p62 (green) antibody, and mounted with DAPI (blue). Scale bar, 50 μm. E, Higher magnified images of triple staining from boxed areas in Fig. 8D. Upper and lower panels show ubiquitin and p62 staining in FKBP8 knockout cells, respectively. Scale bar, 5 μm. F, Ultrastructural analysis of the perinuclear region in sham- and TAC-operated Fkbp8<sup>+/+</sup> and Fkbp8<sup>−/−</sup> mice. Scale bar, 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
of Proteostat fluorescent dye-positive misfolded protein aggregates in FKBP8 knockdown H9c2 myocytes and electron dense deposits in the perinuclear region in the cells and TAC-operated Fkbp8−/− hearts. Proteostat-positive aggregates in FKBP8 knockdown H9c2 cells were not ubiquitinated nor p62-positive. Furthermore, autophagy was not upregulated and the activation of autophagy had no effect on the misfolded protein accumulation in FKBP8 knockdown cells. Thus, it is unlikely that autophagy is involved in the degradation of FKBP8-mediated protein aggregates. Knockdown of FKBP8 induced Protosteat dye-positive protein aggregates without affecting ER stress or cell death, whereas H2O2 treatment in FKBP8 knockdown H9c2 cells accelerated ER stress and cell death without increasing the number of the cells with Protosteat dye-positive protein aggregates. Therefore, protein aggregates themselves in FKBP8 knockdown H9c2 myocytes were not toxic to the cells. However, H2O2 administration resulted in apoptotic cell death, suggesting that FKBP8 knockdown H9c2 cells with Protosteat-positive structures were more susceptible to H2O2 stimuli. H2O2-induced accumulation of misfolded proteins which are not detected by Protosteat dye resulted in ER stress and subsequent caspase-12-mediated apoptosis in FKBP8 knockdown cells. In in vivo, there was no difference in cardiac function or ultrastructure of cardiomyocytes between sham-operated Fkbp8+/+ and Fkbp8−/− hearts, suggesting that compensatory mechanisms prevented the accumulation of misfolded proteins in Fkbp8−/− mice. However, upon pressure overload, Fkbp8−/− mice cannot overcome misfolded protein load, leading to ER stress-induced apoptosis and development of dilated cardiomyopathy, indicating that FKBP8 plays an essential role in the prevention of misfolded protein accumulation and resultant apoptosis in pressure overloaded hearts. It is likely that upregulation of FKBP8 in the heart after TAC is an adaptive mechanism against stress. The precise molecular mechanisms how protein aggregates affect myocardial damage remain to be elucidated.

Chaperones and co-chaperones are essential for protein quality control mechanisms to facilitate protein folding and refolding [4]. Protein misfolding can take place in the ER as well as outside the ER in the cell [32]. Members of the HSP90 family are the most abundant chaperones and highly expressed in cardiomyocytes [4]. We found that FKBP8 binds to HSP90 in cardiomyocytes. FKBP8 is localized to the ER membrane, but almost entirely exposed to the cytosol and contains tetratricopeptide repeat domain (Supplementary Fig. 1A) [9,10], which is known to interact with HSP90 [43]. FKBP8 may be involved in protein-folding machinery interacting with HSP90 at the ER for protein and ER quality control to prevent accumulation of misfolded proteins. Further study will be required to investigate how the interaction of ER protein FKBP8 with cytosolic protein HSP90 coordinates as chaperone machinery in the cardiomyocytes. FKBP8 is reported to promote the maturation of HERG channel and cytosic fibrosis transmembrane conductance regulator (CFTR) [12,44]. However, we could not detect differences in the protein levels of mature HERG or CFTR between TAC-operated Fkbp8+/+ and Fkbp8−/− hearts (Supplementary Fig. 9A and 9B). The substrates of FKBP8 or the components of accumulated protein aggregates in FKBP8-deficient cardiomyocytes remain to be elucidated. In addition, the mechanism to degrade the FKBP8-related protein aggregates requires further investigation.

In summary, deficiency of FKBP8 induced the development of dilated cardiomyopathy. This is presumably due to increased apoptotic cardiomyocyte death and the accumulation of misfolded proteins. It has been reported that FKBP8 plays various roles in cellular and molecular regulation, so it is possible that the function of FKBP8 might be cell- or stress-type specific. In cardiomyocytes, FKBP8-mediated protein quality control is critical to maintain cellular homeostasis against stress. Our findings provide the first evidence that FKBP8 has protective effects in the heart and upregulation of FKBP8 could be a potential therapeutic target for heart failure patients.

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Disclosures

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Appendix A. Supplementary data

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References

[1] C.W. Yancy, M. Jessup, B. Buxkort, J. Butler, D.E. Casey Jr., M.H. Drazner, et al., 2013 ACCF/AHA guideline for the management of heart failure: executive summary: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines, Circulation 128 (2013) 1810–1852.
[2] P.M. McLendon, J. Robbins, Proteotoxicity and cardiac dysfunction, Circ. Res. 116 (2015) 1863–1882.
[3] M.S. Willis, C. Patterson, Proteotoxicity and cardiac dysfunction—Alzheimer’s disease of the heart? N. Engl. J. Med. 363 (2013) 455–464.
[4] M.S. Willis, C. Patterson, Hold me tight: role of the heat shock protein family of chaperones in cardiac disease, Circulation 122 (2010) 1740–1751.
[5] T. Minamino, M. Kitakaze, ER stress in cardiovascular disease, J. Mol. Cell. Cardiol. 48 (2010) 1105–1110.
[6] N. Mizushima, M. Komatsu, Autophagy: renovation of cells and tissues, Cell 147 (2011) 728–741.
[7] K. Okamoto, N. Kondo-Okamoto, Y. Ohsumi, Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy, Dev. Cell 17 (2009) 87–97.
[8] T. Murakawa, O. Yamaguchi, A. Hashimoto, S. Hikoso, T. Takeda, T. Oka, et al., Bcl-2-like protein 13 is a mammalian Atg32 homologue that mediates mitophagy and mitochondrial fragmentation, Nat. Commun. 6 (2015) 7527.
[9] E. Lam, M. Martin, G. Wiederrecht, Isolation of a cDNA encoding a novel human FRK06 binding protein homolog containing leucine tetratricopeptide repeat motifs, Gene 160 (1995) 297–302.
[10] M. Shirane, M. Ogawa, J. Motoyama, K.I. Nakayama, Regulation of apoptosis and neurite extension by FKBP38 is required for neural tube formation in the mouse, Development 131 (2004) 2149–2159.
[11] K. Haupt, G. Jahres, M. Linnert, M. Maestre-Martinez, M. Malesevic, A. Pechstein, et al., FKBP8 catalytic domain binds to Bcl-2 via a charge-sensitive loop, J. Biol. Chem. 282 (2007) 23509–23516.
[12] B.H. Choi, L. Feng, H.S. Yoon, FKBP38 protects Bcl-2 from caspase-dependent degradation, J. Biol. Chem. 285 (2010) 9770–9779.
[13] A. Nakai, O. Yamaguchi, S. Hikoso, T. Takanoe, M. Taniike, et al., FKBP8 is a nega-
receptor 9 prevents cardiac rupture after myocardial infarction in mice independently of inflammation, Am. J. Physiol. Heart Circ. Physiol. 311 (2016) H1485–H1497.

[22] M. Tatei, K. Nishida, S. Omiya, E. Zarriqzadeh, T. Misaka, R. Kitazume-Tanaka, et al., mTOR hyperactivation by ablation of tuberous sclerosis complex 2 in the mouse heart induces cardiac dysfunction with the increased number of small mitochondria mediated through the down-regulation of autophagy, PLoS One 11 (2016) e0152628.

[23] D. Shen, J. Coleman, E. Chan, T.P. Nicholson, L. Dai, P.W. Sheppard, et al., Novel cell- and tissue-based assays for detecting misfolded and aggregated protein accumulation within aggresomes and inclusion bodies, Cell Biochem. Biophys. 60 (2011) 173–185.

[24] N.N. Noda, H. Kumeta, H. Nakatogawa, K. Satoo, W. Adachi, J. Ishii, et al., Structural basis of target recognition by Atg8/LC3 during selective autophagy, Genes Cells 13 (2008) 1211–1218.

[25] J. Kim, J.J. Lemasters, Mitochondrial degradation by autophagy (mitophagy) in GPF-LC3 transgenic hepatocytes during nutrient deprivation, Am. J. Physiol. Heart Circ. Physiol. 300 (2011) C308–C317.

[26] T. Oka, S. Hikoso, O. Yamaguchi, M. Tatei, T. Takada, T. Tamai, et al., Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure, Nature 485 (2012) 251–255.

[27] A. Shirakabe, P. Zhai, Y. Ikeda, T. Saito, Y. Maejima, C.-P. Hsu, et al., Drp1-dependent mitochondrial autophagy plays a protective role against pressure overload-induced mitochondrial dysfunction and heart failure, Nature 485 (2012) 251–255.

[28] D. Ebrahimi-Fakhari, A. Safifari, L. Wahlster, A. DiNardo, D. Turner, T.L. Lewis Jr.et al., Impaired mitochondrial dynamics and mitophagy in neuronal models of tuberous sclerosis complex, Cell Rep. 17 (2016) 1053–1070.

[29] Z. Bhujabal, A.B. Birgisdottir, E. Sjottem, H.B. Brenne, A. Overvatn, S. Habisov, et al., Identification of a candidate therapeutic autophagy-inducing peptide, Nature 494 (2013) 201–206.

[30] P. Tannous, H. Zhu, A. Nemchenko, J.M. Berry, J.L. Johnstone, J.M. Shelton, et al., Intracellular protein aggregation is a proximal trigger of cardiomyocyte autophagy, Circulation 117 (2008) 3070–3078.

[31] F. Edlich, M. Weivad, F. Erdmann, J. Fanghanel, F. Jarzczowski, J.U. Rahfeld, et al., Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin, EMBO J. 24 (2005) 2688–2699.

[32] A. Saita, M. Shirane, K.I. Nakayama, Selective escape of proteins from the mitochondria during mitophagy, Nat. Commun. 4 (2013) 1410.

[33] Z. Osawa, K. Tanaka, K. Terasaki, H. Endo, N. Ueno, M. Ueda, et al., mTOR hyperactivation by ablation of tuberous sclerosis complex 2 induces myocardial dysfunction with the increased number of small mitochondria mediated through the down-regulation of autophagy, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) C308–C317.

[34] A. Buchberger, B. Bukau, T. Sommer, Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms, Mol. Cell. Cell Biol. 40 (2010) 238–252.

[35] Y. Maejima, S. Kyo, P. Zhai, T. Liu, H. Li, A. Ivesa, et al., Mrl1 inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2, Nat. Med. 19 (2013) 1478–1488.

[36] S. Parkiv, T.H. Clausen, T. Lamark, A. Beech, J.A. Bruun, H. Outzen, et al., p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, J. Biol. Chem. 282 (2007) 24131–24145.

[37] S. Shoji-Kawata, R. Sampaer, M. Leveno, G.R. Campbell, Z. Zou, L. Kinch, et al., Expression of R120G-alphaB-crystallin causes aberrant desmin and alphaB-crystallin aggregation and cardiomyopathy in mice, Circ. Res. 89 (2001) 84–91.

[38] R.R. Kopito, Aggresomes, inclusion bodies and protein aggregation, Trends Cell Biol. 10 (2000) 524–530.

[39] X. Wang, H. Otsuka, R. Klevitsky, M. Nieman, J. Lorenz, et al., Expression of R120G-alphaB-crystallin causes aberrant desmin and alphaB-crystallin aggregation and cardiomyopathy in mice, Circ. Res. 89 (2001) 84–91.

[40] A. Sanbe, H. Otsuka, J.E. Saffitz, C.G. Glabe, R. Kayed, A. Maloyan, et al., Desmin-related cardiomyopathy in transgenic mice: a cardiac amyloidosis, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 10132–10136.

[41] M.S. Bhuiyan, J.S. Pattison, H. Osinska, J. James, J. Gulick, P.M. McLendon, et al., Enhanced autophagy ameliorates cardiac proteinopathy, J. Clin. Invest. 123 (2013) 5284–5297.

[42] L. Rabinowitch, A. Brinker, G. Bourenkov, S. Pogorarov, L. Melber, H. Bartunik, et al., Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine, Cell 89 (1997) 1097–1108.

[43] Y.K. Banasavadi-Siddegowda, J. Mai, Y. Fan, S. Bhattacharya, D.R. Giovannucci, E.R. Sanchez, et al., FKBP38 peptidylprolyl isomerase promotes the folding of cystic fibrosis transmembrane conductance regulator in the endoplasmic reticulum, J. Biol. Chem. 286 (2011) 43071–43080.