이학박사학위논문

미토콘드리아의 분열과 분해에서 FKBP8의 역할에 관한 연구

A study on the role of FKBP8 in mitochondrial fission and degradation

2017년 8월

서울대학교 대학원

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A study on the role of FKBP8 in mitochondrial fission and degradation

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by

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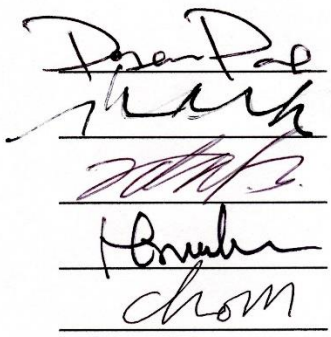

ABSTRACT

A study on the role of FKBP8 in mitochondrial fission and degradation

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Mitochondria change its shape continuously through fission and fusion which help to maintain functional mitochondria under metabolic or environmental stress condition. Dysfunction of such mitochondrial fusion and fission dynamics and degradation of mitochondria evokes failure of mitochondrial quality control and has been linked to several human diseases. Thus, identification of novel factor involved in mitochondrial dynamics and mitophagy are important and will provide mechanistic insight into mitochondria quality control. Here, I report that

FKBP8, a FK506-binding protein 8 which is a member of the immunophilin protein family, has a critical role in mitochondrial fission and degradation. Ectopic expression of FKBP8 increased the numbers of cells showing mitochondrial fragmentation and induced drastic co-localization of GFP-LC3B as well as GFP-GABARAPL1 or GABARAPL2 with the mitochondria Deletion-mapping and mutagenesis analysis revealed that the N-terminal region was required for FKBP8-mediated LC3 recruitment onto mitochondria. Especially, the LIR#1 ($_{24}$FEVL$_{27}$) motif of FKBP8 was critical for the binding to LC3. Interestingly, ectopic expression of FKBP8 induced mitochondrial fragmentation and this ability was unique to FKBP8 among FKBP family. Conversely, knockdown of FKBP8 expression by RNA interference increased the volume and number of mitochondria. Especially, enlarged mitochondria was typically observed under electron microscope. FKBP8-induced mitochondrial fragmentation occurred independently of Drp1, BNIP3 and NIX, well known mitochondrial fission/mitophagic factors, but was abolished by mutation in the LIR#2 ($_{93}$WLDI$_{96}$). Further, I found that FKBP8 interacted with FIS1, a mitochondrial fission factor. But deletion in the LIR#2 ($_{93}$WLDI$_{96}$) or deletion in TPR domain of FKBP8 reduced the binding of FKBP8 to FIS1. In addition, knockdown of FKBP8 expression attenuated mitochondrial degradation under

hypoxia. Together, these results suggest that FKBP8 mediates mitochondrial dynamics and degradation by binding to FIS1 through the LIR#2/TPR domain and perform mitophagic event by binding to LC3 through the LIR#1 motif.

Key Words: Mitophagy, Autophagy, Mitochondrial fission, LIR, FKBP8, LC3, FIS1

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ABBREVIATION

BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
Drp1	Dynamin-related protein 1
FIS1	Mitochondrial fission 1 protein
FKBD	FK506 binding domain
FKBP	FK506 binding protein
FKBP8	FK506 binding protein 8
FUNDC1	FUN14 domain containing 1
G418	Geneticin
GABARAPL1	GABA type A receptor associated protein like 1
GABARAPL2	GABA type A receptor associated protein like 2
LC3	Microtubule associated protein 1 light chain 3
LIR	LC3 interacting region
MFN1	Mitofusin-1
NIX	BCL2/adenovirus E1B interacting protein 3-like

PARL	Presenilin associated rhomboid like
PINK1	PTEN induced putative kinase 1
PPiase	Peptidylprolyl isomerase
ROS	Reactive oxygen species
TIMM23	Translocase of inner mitochondrial membrane 23
TM	Transmembrane
TOMM20	Translocase of outer mitochondrial membrane 20
TPR	Tetratricopeptide repeat

INTRODUCTION

Mitochondria are important intracellular organelles which regulate ATP level, Ca2+ store, apoptosis, and formation of protein, carbohydrate and lipids (Bratic & Trifunovic, 2010; Ballard & Youngson, 2015). In order to maintain the healthy mitochondria, protein degradation and chaperones are responsible for the quality of the protein at protein level, and mitochondrial fission/fusion and degradation of damaged mitochondria are responsible for the quality of mitochondrion at organelle level. When damages are accumulated over capacity of mitochondrial quality control, impairment of normal function of mitochondria induces more reactive oxygen species (ROS), fail to produce metabolites and induces eventually cell death with cytochrome c release. Because of these features, defects in mitochondria have been reported to induce many diseases, such as aging-related diseases associated with metabolism, muscle, and neurodegenerative disease, such as Parkinson's disease (Duchen, 2004; Nunnari & Suomalainen, 2012).

Morphology of mitochondria are not monolithic but network and

continuously undergo fusion and fission called ‘mitochondrial dynamics’. The mitochondrial fusion occurs to compensate for the normal function of mitochondria and mitochondrial fission divides into healthy and damaged daughter mitochondrion. It has been reported that fusion of mitochondrial outer membrane correlates with forming of MFN1 and 2 oligomer, and fusion of inner membrane correlates with forming of OPA1 oligomer. Mitochondrial fission is important for the formation of daughter mitochondrion to migrate and degrade the mitochondria. Drp1 plays an important role in the mitochondrial fission. Drp1 binds mitochondrial outer membrane proteins, such as MFF and FIS1, and induces mitochondrial fission by forming a ring-like oligomer. When mitochondria fail to maintain normal function through mitochondrial dynamics, damaged mitochondria are degraded by selective degradation of mitochondria through autophagy (Archer, 2013).

Autophagy is the process of degradation using lysosome. There are three types of autophagy, macroautophagy, microautophagy and chaperone-mediated-autophagy (CMA). Macroautophagy is a process of forming an autophagosome vesicle with a double membrane structure. PI3K class III induces PI3P to produce isolation membrane,

membrane expansion by using two-ubiquitin-like systems and maturation of isolation membrane eventually form autophagosome to isolate the cytosolic compartments from the cytosol. Sequestered cargo by autophagosome is subsequently degraded through lysosome. On the other hand, microautophagy degrades cytosolic cargo through direct engulfing through lysosome without autophagosome formation. Chaperone-mediated autophagy is the degradation process of selectively recruited proteins through chaperone proteins. Autophagy degrades various target proteins and organelles, including aggregated proteins, membrane, ER, peroxisome, proteins, invaded bacteria, viruses and mitochondria. Especially, specific degradation of targets via autophagy is called selective autophagy (Glick, Barth, & Macleod, 2010).

Mitophagy is a type of selective autophagy that refers to the selective degradation of mitochondria via autophagy. Mitophagy occurs during starvation, hypoxia, depolarized mitochondrial degradation and maturation of erythrocytes. Compared to others, the mechanism of damaged mitochondrial degradation, which is depolarized, is relatively well studied. PINK1 and Parkin have critical role in the degradation of depolarized mitochondria. PINK1 is continuously degraded by PARL

protease in the matrix of healthy mitochondria. But when mitochondria are depolarized, the activity of PARL is lowered so that PINK1 is not degraded any more in the mitochondria, accumulates on outer membrane of mitochondria and recruits Parkin, an E3 ligase, to damaged mitochondria. Parkin ubiquitinates mitochondrial proteins and these ubiquitination is recognized by adapter proteins, such as P62, NDP52 and OPTN. These adapter proteins selectively eliminate damaged mitochondria by recognizing autophagosomes through its LC3-interacting-Region (LIR) motif (Youle & Narendra, 2011).

In an alternative pathway, adapter proteins, such as NIX, FUNDC1 and cardiolipin, bind directly to LC3 via LIR motif under various situations. NIX recognizes autophagosomes directly through its LIR in the maturation process of red blood cells and FUNDC1 is phosphorylated by SRC kinase under hypoxia, thereby enhancing LC3 recognition for selective degradation of mitochondria. It was also reported that cardiolipin is located in the inner membrane of the mitochondria, but when the mitochondria are damaged, it migrates to the outer membrane of the mitochondria and induces mitophagy by recognizing LC3 (Ni, Williams, & Ding, 2015). However, specific role and signaling of these mitophagic factors under physiological and

pathological conditions are largely unknown. Moreover, identification of additional factors functioning in mitophagy is important to better understand mitochondrial quality control.

FKBP8 is one of the FK506-binding domain-containing family. FK506 binding domain has peptidylprolyl isomerase (PPiase) activity and is inhibited by immunosuppressant drug, such as FK506, Rapamycin and cyclosporine A. FKBP8 consists of FK506-binding domain, three tetratricopeptide repeat (TPR) domain, calmodulin binding region and transmembrane domain. It is believed that FKBP8 is exposed to cytosol with the cytosolic side anchored to the outer membrane of mitochondria through the C-terminal transmembrane domain (Shirane & Nakayama, 2003; Thomson, Bonham, & Zeevi, 1995). Unlike other FKBP family, PPiase activity of FKBP8 is constitutively inactive and only active when $Ca2^{+}$-bound calmodulin binds (Haupt et al., 2012). Also, FKBP8 shows anti-apoptotic effect through binding to Bcl2 and Bcl-xL and inhibition of mTORC1 (Shirane et al., 2003). Recently, it was reported that FKBP8 escapes from mitochondria to ER in a Parkin-dependent manner when cells were treated with CCCP (Saita, Shirane, & Nakayama, 2013). However, a role of FKBP8 in mitophagy is not known at all.

In this study, I identified a novel mitophagy factor, FKBP8, from cell-based functional screening using cDNA expression library, and characterized its novel role in mitochondrial dynamics and mitophagy.

RESULTS

Establishment of cell-based screening assay to isolate mitophagy regulator

To identify a novel mitophagy regulator, I decided to employ gain-of-functional cell-based functional screening assay using cDNA expression library. I made a hypothesis that, if a mitophagy regulator induced mitochondrial damage for the induction of mitophagy or affected mitochondrial localization of specific autophagy/mitophagy marker in cell-based assay, the cDNA encoding the mitophagy regulator could be identified from cDNA expression library (Fig. 1). Thus, I first established mitophagy cell-based assay. Chang liver cells were transfected with GFP-LC3B, an autophagy marker which binds to autophagy substrate to recruit it into autophagosome, and Mito-RFP .Then treated the cells with mitophagy-inducing reagent CCCP. I observed that GFP-LC3 dots were recruited into the mitochondria under fluorescence microscope (data not shown), showing that subcellular localization of GFP-LC3B onto the damaged mitochondria. As other control assay, I transfected Chang liver cells with GFP-LC3B and NIX, a mitophagy regulator found in white blood cells, and found recruitment of GFP-LC3B onto mitochondria

(data not shown).

Second, to isolate new mitophagy regulators, I decided to primarily focus on the role of mitochondrial proteins in the mitophagy and because until now many key regulators, such as PINK, NIX and BNIP3 (Ding & Yin, 2012) are located in the mitochondria. Instead of siRNA library, I chose cDNA expression library because gain-of-functional screening might sometimes provide new opportunity to isolate unusual regulators. Among about 800 mitochondrial proteins, gateway cloning was performed to prepare human cDNAs encoding mitochondrial proteins in mammalian expression vector. Some cDNAs in mammalian expression were also prepared by DNA sequencing analysis project and provided by CRIBB as well. Total 494 cDNA clones were prepared and isolated by affinity column for the screening in cell-based assay (Fig. 2).

Identification of FKBP8 as a GFP-LC3B recruiting factor to mitochondria

Then, I performed functional screening using the 494 cDNA clones. Chang liver cells were plated in 48-well culture plates one day before and then were transiently cotransfected with GFP-LC3B and 494 cDNA encoding mitochondrial proteins. NIX was utilized as a positive control in the functional screening. From primary screening under fluorescence

microscope, 40 putative positive clones that affected GFP-LC3B dot formation and subcellular localization were isolated. After secondary screening, 17 putative positive clones were consistently active to increase GFP-LC3B dot formation in the transfected cells (Fig. 3A). Many of them are involved in the metabolism, such as amino acid synthesis, glycerolipid metablolism and ketogenesis etc., and are also linked to human disease, such as mood disorder, kidney disease and tumor (Fig. 4).

Among them, FKBP8, a FK506-binding protein 8, showed the highest impact on the formation of GFP-LC3B dots and was even better to increase the number of GFP-LC3B dots than the positive control NIX did (Fig. 3A). Interestingly, overexpression of FKBP8 or PTRH2 induced change in the subcellular localization of GFP-LC3B. Compared to the diffused pattern of GFP-LC3B in control cells, GFP-LC3B seemed to colocalize with mitochondria following FKBP8 overexpression (Fig. 3B). To confirm this, Chang liver cells were co-transfected with FKBP8, GFP-LC3B and Mito-RFP and examined mitochondrial localization of GFP-LC3B. As expected, ectopic expression of FKBP8 induced drastic colocalization of GFP-LC3B and Mito-RFP (Fig. 5A). In the LC3 family, there are GABALAPL1 and L2 as well as LC3. Thus, I tested whether or not FKBP8 affected mitochondrial localization of GABALAPL1 and

L2. From the same experiments, I found similar results showing that GABALAPL1 and L2 were also recruited onto the mitochondria following FKBP8 overexpression (Fig. 5B).

FKBP8 interacts with GFP-LC3B through its N-terminal to recruit it onto mitochondria

Because FKBP8 is a mitochondrial protein which is located on the outer membrane (Shirane & Nakayama, 2003; Thomson, Bonham, & Zeevi, 1995), I hypothesized that FKBP8 might interact with LC3 to recruit it onto the mitochondria. To test this hypothesis, I decided to determine FKBP8 domain responsible for the recruitment of GFP-LC3B onto the mitochondria. Based on the previous report, it is known that FKBP8 contains FK506-binding domain (FKBD) and 3 tetratricopeptide (TPR) domain in the middle and transmembrane region (TM) at the C-terminus (Fig. 6A) Lam, Martin, & Wiederrecht, 1995; Pedersen, Finsen, Celis, & Jensen, 1999; Shirane & Nakayama, 2003). I generated serial 5 deletion mutants of FKBP8, including FKBP8-dN mutant lacking the N-terminus, FKBP8-dF lacking FKBD, FKBP8-dNF lacking the N-terminus and FKBD, and FKBP8-dNTF lacking the N-terminus, FKBD and TPR domain (Fig. 6A).

From similar expression analysis, I found that FKBP8 WT was also able

to induce colocalization of GFP-LC3B and Mito-RFP in HeLa cells lacking Parkin (Fig. 6B). Like FKBP8 wild-type, ectopic expression of FKBP8-dF lacking FKBD was potent to induce colocalization of GFP-LC3B and Mito-RFP. On the contrary, ectopic expression of FKBP8-dN mutant lacking the N-terminus, FKBP8-dNF lacking the N-terminus and FKBD, or FKBP8-dNTF lacking the N-terminus, FKBD and TPR domain failed to do so (Fig. 6B). All of these mutants have a common deletion, the N-terminal region. As a result, I found that the N-terminal region comprising 103 amino acids is important for recruiting GFP-LC3 onto the mitochondria (Fig. 6A). However, this N-terminal region of FKBP8 has no reported function.

Furthermore, I assessed a possibility that FKBP8 ability to affect colocalization of GFP-LC3B with mitochondria is due to its binding to GFP-LC3B. The results from immunoprecipitation analysis using anti-HA antibody showed that GFP-LC3B was found in the immunoprecipitated containing HA-FKBP8 or FKBP8-dF lacking FKBD. On the contrary, GFP-LC3B was not immunoprecipitated together with FKBP8-dN mutant lacking the N-terminus, FKBP8-dNF lacking the N-terminus and FKBD, or FKBP8-dNTF lacking the N-terminus, FKBD and TPR domain (Fig. 7, upper). The deletion mutants were all expressed in the transfected cells (Fig. 7, lower). These results

indicate that FKBP8 interacts with GFP-LC3 and the N-terminal region is important for binding to GFP-LC3B. Together, I believe that FKBP8 induces colocalization of GFP-LC3B on the mitochondria probably through protein-protein interaction.

FKBP8 interacts with GFP-LC3B through LIR motif in the N-terminal region

To further find critical element responsible for the binding of FKBP8 to GFP-LC3B, I compared amino acid sequence in FKBP8 with LIR motif. A LC3-interacting region (LIR) motif is known to be critical for the binding to LC3. From sequence comparison, I found a classical LIR motif, W/Y/F xx I/L, in the N-terminal region of FKBP8. Interestingly, I found two LIR motives, LIR#1 (F25-E-V-L27) and LIR#2 (W93-L-D-I96), in the N-terminal region of FKBP8 (Fig. 8A). Two LIR motives are about 70 residues apart from each other and are highly conserved among different species, including human, bovine, mouse and rat (Fig. 8B).

To determine which of the two LIR motives present in FKBP8 is actually important for the binding to GFP-LC3B and its recruitment onto mitochondria, three kinds of FKBP8 LIR point mutants were generated with site-directed mutagenesis using synthetic oligonucleotides containing mutation in the corresponding residues. FKBP8 LIR#1 F24A-

L27A mutant has replacement in the conserved 24th Phe and 27th Leu residues with Ala, FKBP8 LIR#2 W93A-I96A mutant has replacement in the 93th Trp and 96th Ile with Ala, and FKBP8 LIR#1+#2 F24A-L27A-W93A-I96A double mutant has mutation in all of these residues (Fig. 8B).

Using these FKBP8 mutants, I addressed whether FKBP8 LIR motives are active to recruit GFP-LC3B onto the mitochondria. From ectopic expression analysis, I found that FKBP8 LIR#2 W93A-I96A mutant could recruit GFP-LC3B on the mitochondria as much as FKBP8 WT did. Unlike FKBP8 LIR#2 W93A-I96A mutant, however, FKBP8 LIR#1 F24A-L27A mutant lost the ability to recruit GFP-LC3B on the mitochondria. Similarly, FKBP8 LIR#1+#2 F24A-L27-W93A-I96A double mutant also lost the ability (Fig. 9A). Quantification of the colocalization showed that mutation in FKBP8 LIR#1 completely abolished the recruitment of GFP-LC3B onto the mitochondria (Fig. 9B). Accordingly, the results from immunoprecipitation assay revealed that FKBP8 LIR#2 W93A-I96A mutant could bind to LC3 but FKBP8 LIR#1 F24A-L27A mutant and FKBP8 LIR#1+#2 F24A-L27-W93A-I96A double mutant could not (Fig. 9C). The results suggest that FKBP8 LIR#1, but not FKBP8 LIR#2, is essential for the binding to and mitochondrial recruitment of LC3.

FKBP8 affects mitochondrial fragmentation and fission

In addition to mitochondrial recruitment of LC3, it was very interesting to note that a number of GFP-LC3B puncta was robustly increased following FKBP8 overexpression. Thus, I decided to carefully examine mitochondria morphology with high magnitude under fluorescence microscope. Surprisingly, I observed that mitochondria in Chang liver and HeLa cells overexpressing FKBP8 were fragmented compared to control cells (Fig. 10A). Under this condition, ER was intact (data not shown). The fragmentation of mitochondria was similarly observed in the cells overexpressing NIX. This effect of FKBP8 on mitochondria fragmentation was similar as NIX in Chang liver cells but bigger than NIX in HeLa cells (Fig. 10B).

Then, I reduced the expression of FKBP8 HeLa cells using shRNA and examined the role of FKBP8 on the dynamics of mitochondria in detail. After selection with G418 for 2 weeks, I confirmed reduced expression of FKBP8 in HeLa cells (HeLa-shFKBP8-Mixed) with western blot analysis (Fig. 11B). HeLa-shFKBP8-Mixed cells were then stained with Mitotracker green and mitochondrial morphology of the cells was examined under fluorescence microscope. Compared to those of HeLa-Neo-Mixed cells, mitochondria in HeLa-shFKBP8-Mixed cells were enlarged with long length (Fig. 11A). Western blot analysis showed that

levels of TOMM20 and TIMM23, outer and inner mitochondria membrane proteins, respectively, in HeLa-shFKBP8-Mixed cells were increased compared to HeLa-Neo-Mixed cells (Fig. 11B).

Moreover, I examined detail morphology of the mitochondria in HeLa-shFKBP8-Mixed cells using electron microscope. Compared to dense and compact mitochondria observed in HeLa-Neo-Mixed cells, the mitochondria in HeLa-shFKBP8-Mixed cells were enlarged in size (Fig. 12A). Apparently, cellular area occupied by the mitochondria was bigger in HeLa-shFKBP8-Mixed cells (Fig. 12B). In addition, I could observe unpacked and aligned cristae in the matrix of mitochondria in HeLa-shFKBP8-Mixed cells. All together, these results suggest that FKBP8 is crucial for the control of mitochondrial dynamics, especially for mitochondrial fission.

Of FKBP family, FKBP8 only affects mitochondrial dynamics

Because FKBP8 belongs to FKBP family comprising more than 15 different FKBP proteins, I tested the ability of other FKBP proteins to affect mitochondrial dynamics. Most of them show specific subcellular localization, including ER, nucleus, mitochondria, membrane and cytosol, and display diverse function in many cellular signaling (Fig. 13A) (Chen, Liu, & Massague, 1997; Nigam et al., 1993; Jin & Burakoff,

1993; Mamane, Sharma, Petropoulos, Lin, & Hiscott, 2000; Toneatto et al., 2013; Takaoka, Ito, Miki, & Nakanishi, 2017; W. Zhang, Zhang, Xiao, Yang, & Zhoucun, 2007; Zhang et al., 2004; Haupt et al., 2012; Okamoto et al., 2008; Hutt et al., 2012; Shadidy et al., 1999; Schwarze et al., 2013; Lin et al., 2013; Nelson et al., 2015; McKeen et al., 2010; McKeen et al., 2008). Among them, I collected 11 FKBP cDNAs and tested their effects on mitochondrial morphology. From co-expression analysis, I found that the mitochondrial fragmentation was induced only by the overexpression of FKBP8 but not by other FKBP family, including FKBP1A, 2, 3, 4, 6, 7, 10, 11, 14 and FKBPL. It is worthwhile to note that FKBP4 is also located in the mitochondria like FKBP8 but did not affect mitochondrial fragmentation (Fig. 13B). Thus, while structural similarity among FKBP family exists, it is only FKBP8 that regulates mitochondrial fragmentation among FKBP family.

FKBP8 LIR#2, not FKBP8 LIR#1, is important for mitochondrial fragmentation

The abilities of FKBP8 to recruit LC3 on the mitochondria and to induce mitochondrial fragmentation are likely separate and may result from distinct domain of FKBP8. Thus, I decided to identify the domain of FKBP8 responsible for mitochondrial fragmentation by using take

advantage of FKBP8 deletions and mutants. As seen in LC3 recruitment assay, examination of mitochondrial morphology revealed that FKBP8 dN, FKBP8 dNF and FKBP8 dNFT mutants, but not FKBP8 dF, failed to induce mitochondrial fragmentation (Fig. 14A). All of FKBP8 dN, FKBP8 dNF and FKBP8 dNFT mutants are lacking the N-terminus, suggesting that the same N-terminal region is important for mitochondrial fragmentation.

I further narrowed down the FKBP8 region responsible for mitochondrial fragmentation using FKBP8 LIR mutants. Interestingly, the fluorescence microscopy revealed that FKBP8 LIR#1 F24A-L27 mutant which lost the ability of LC3 recruitment onto the mitochondria could still induce mitochondrial fragmentation (Fig. 14B, upper). On the contrary, FKBP8 LIR#2 W93A-I96A mutant and FKBP8 LIR#1+#2 F24A-L27-W93A-I96A double mutant showed little effect on mitochondrial fragmentation (Fig. 14B, lower). Together, the results suggest that FKBP8 LIR#2, not FKBP8 LIR#1, plays an essential role in mitochondrial fragmentation and that FKBP8 LIR#1 and 2 play distinct role in mitophagy and mitochondrial dynamics, respectively.

FKBP8 induces mitochondrial fragmentation in the absence of Drp1

For the mitochondrial fission, although there are some exceptions

(Stavru, Palmer, Wang, Youle, & Cossart, 2013; Ishihara et al., 2009), the recruitment of Drp1 to mitochondria for mitochondrial fission is well established (Reddy et al., 2011). Therefore, I examined whether or not Drp1 was required for FKBP8-induced mitochondrial fragmentation. Overexpression of FKBP8 effectively induced mitochondrial fragmentation in wild-type mouse embryo fibroblasts (WT MEFs) (Fig. 15A). As seen in WT MEFs, overexpression of FKBP8 could also induce mitochondrial fragmentation in Drp1 KO MEF cells. No difference in the ratio of fragmented mitochondria was observed between WT and Drp1 KO MEF (Fig. 15B). In addition, overexpression of Drp1 K38A, a dominant negative of Drp1, did not affect FKBP8-induced mitochondrial fragmentation (data not shown). These result suggest that FKBP8 induces mitochondrial fragmentation independently of Drp1.

Overexpression of FKBP8 induces mitophagy in BNIP3 and NIX knockout cells

Most of LIR motif-containing proteins, including FKBP8, are target LC3 on autophagosome and subsequently deliver cargos for degradation through lysosome (Wild, McEwan, & Dikic, 2014). Then, I further investigated downstream factors of FKBP8-mediated degradation of mitochondria. To monitor mitophagy, Mito-Keima which shows red

fluorescence signal in low pH and green fluorescence signal in mitochondrial pH was cotransfected together with HA-FKBP8 into Drp1, BNIP3/NIX or FIP200 knockout cells. BNIP3/NIX were previously reported as mitophagy factor (Zhu et al., 2013; Novak et al., 2010) and FIP200 is essential ULK1 complex factor to process autophagy (Hara et al,. 2008). Consistent to that of Drp1 KO MEFs, Drp1 expression induced mitochondrial fragmentation in Drp1 KO HeLa cells (Fig. 16). Interestingly, HA-FKBP8 expression also induced mitochondrial fragmentation in BNIP3/NIX double KO (DKO) Hela cells, but not in FIP200 KO cells (Fig. 16). All these data suggest that FKBP8 is a novel factor which mediates mitochondrial degradation independently of Drp1, BNIP3 and NIX, and needs macroautophagy activity.

FKBP8 interacts with FIS1 through LIR#2 and TPR domains

I was then eager to identify a downstream mediator of FKBP8 in mitophagy. I focused on FIS1 because it is located on the outer membrane of mitochondria as FKBP8 and is one of the factors responsible for mitochondrial fission (Mozdy, McCaffery, & Shaw, 2000). First, I tested whether FKBP8 binds to FIS1 using immunoprecipitation assay. Interestingly, the results revealed that HA-FKBP8 could interact with FIS1-GFP very well (Fig. 17A). Then, I

decided to map the domain responsible for this interaction. The results from immunoprecipitation analysis using FKBP8 deletion mutants showed that FIS1-GFP bound to HA-FKBP8 but weakly to FKBP8 d54-93 mutant containing LIR#1 but lacking LIR#2 (Fig. 17B). More, FIS1-GFP did not bind to FKBP8 deletion mutants, FKBP8-dFT and -TPR lacking TPR domain. These results suggest that FIS1 binds to FKBP8 through LIR#2 and TPR to mediate mitochondrial fission.

Knockdown of FKBP8 reduces mitochondrial fragmentation and degradation in hypoxia

To dissect the role of FKBP8 in physiologic and pathologic signaling, the role of FKBP8 in signaling were examined. The effects of many signaling, including CCCP, ROS and starvation, on the mitophagy and mitochondrial fragmentation were examined but no significant effect was observed (data not shown). Unlike CCCP, however, a significant role of FKBP8 was found in hypoxia. Under hypoxia for 48 h, the mitochondrial fragmentation was evident in HeLa-Neo-Mixed cells. On the other hand, mitochondrial fragmentation was apparently reduced in HeLa-shFKBP8-Mixed cells (Fig. 18A, B). Analysis of mitochondrial fragmentation with quantification for 24 and 48 h revealed that tubular and intermediate forms of mitochondria was dramatically increased but

fragmented form of mitochondria decreased 3-fold at 24 h and 30% at 48 h in HeLa cells by FKBP8 knockdown (Fig. 18C, D), suggesting that FKBP8 is essential in mitochondrial fragmentation under hypoxia.

Accordingly, western blotting revealed that levels of TOMM20 and TIMM23 mitochondrial proteins was largely reduced at 96 h in HeLa-Neo-Mixed cells. However, these reduction was apparently alleviated in HeLa-shFKBP8-Mixed cells (Fig. 19A). Conversely, overexpression of FKBP8 accelerated the degradation of TOMM20 and TIMM23 under hypoxia (Fig. 19B). In fact, analysis with electron microscopy also supported the protective role of FKBP8 against hypoxic damage. Compared to those of HeLa-Neo-Mixed cells, mitochondria in HeLa-shFKBP8-Mixed cells were enlarged under normoxia and remained in a larger form and appeared not to be removed by mitophagy.

Fig. 1. Schematic diagram for the screening of mitophagy modulator. Mitophagy is a selective degradation of mitochondria through autophagy. The damaged and ubiquitin-tagged mitochondria are recognized by autophagic markers, such as LC3, during mitophagy. To find out novel mitophagy/LC3-recruiting factors, cell-based assay employing GFP-LC3B and Mito-RFP was established and tested for the effect of each mitochondrial protein on mitophagy.

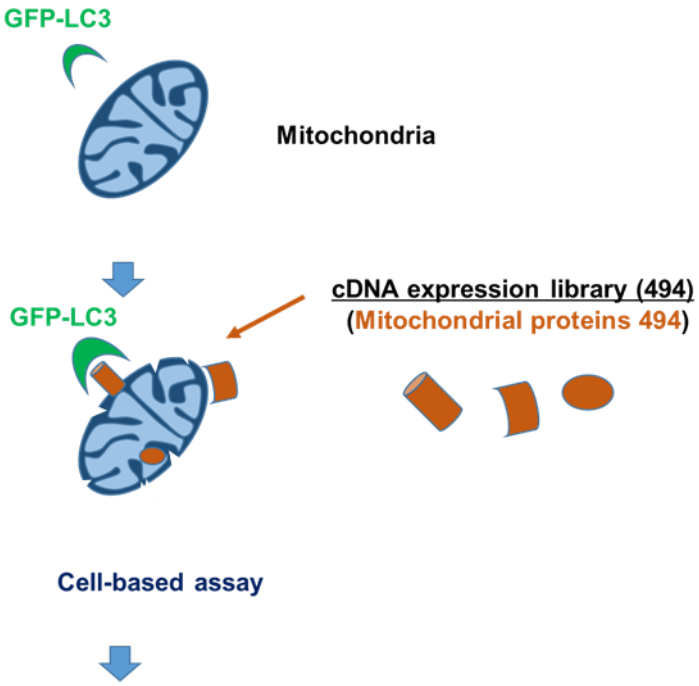
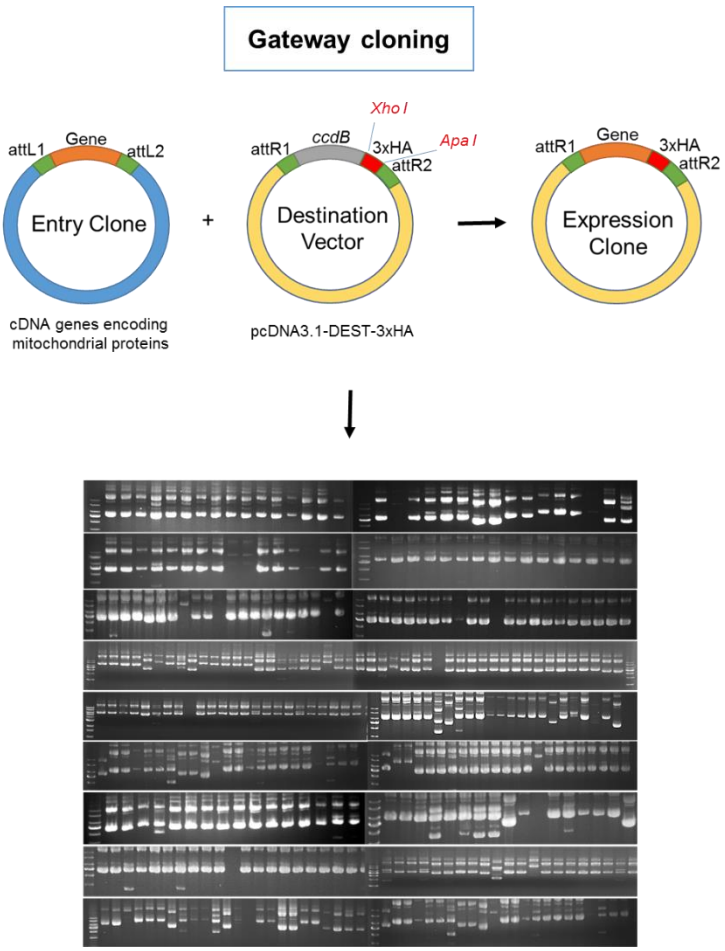

GFP-LC3
Mitochondria
GFP-LC3
cDNA expression library (494)
(Mitochondrial proteins 494)
Cell-based assay
Mitophagy/LC3-recruiting factor

Fig. 2. Preparation of cDNA expression library encoding mitochondrial proteins by using gateway cloning.

Gateway cloning was performed to prepare the cDNA expression library for mitochondrial protein. Genes in Entry clones was transferred to pcDNA3.1-DEST-3xHA destination vector using purified recombinase. Total 494 expression clones are prepared for the cDNA expression library and confirmed by agarose gel electrophoresis.



cDNA expression library
(Mitochondrial proteins)

494 genes

Fig. 3. The results of cell-based functional screening with GFP-LC3B. (A) The results of cell-based GFP-LC3B puncta screening. Chang liver cells were transiently transfected with GFP-LC3B and each of the cDNA expression library. After 24 h, GFP-LC3B puncta-positive cells were counted under fluorescence microscope and its percentages were calculated. Bars represent mean ± SD (n = 3). (B) Typical images of the cells showing enhanced GFP-LC3B puncta by PTRH2 or FKBP8 overexpression.

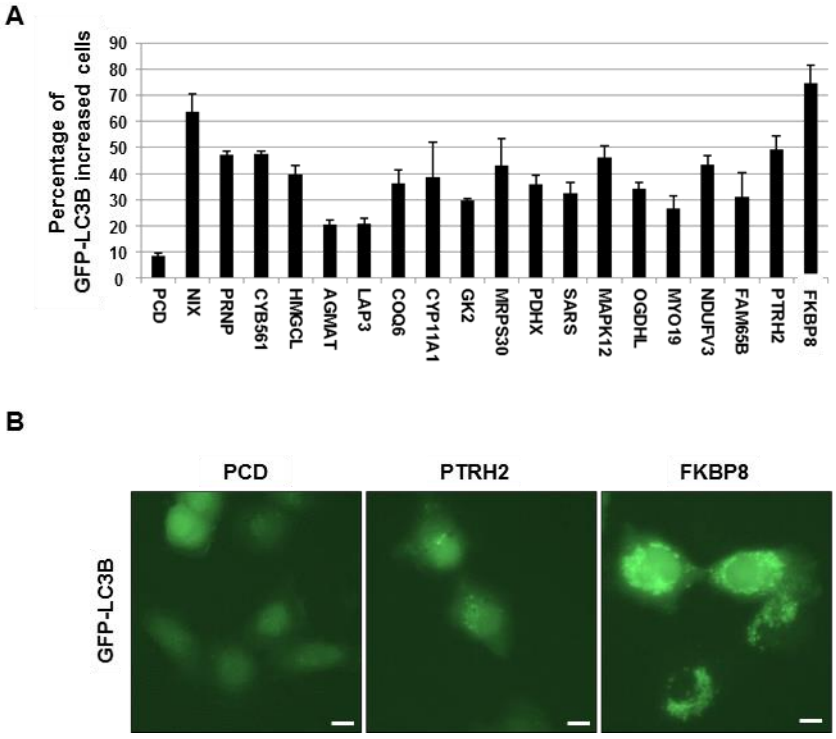

A
Percentage of GFP-LC3B increased cells
90
80
70
60
50
40
30
20
10
0
PCD
NIX
PRNP
CYB561
HMGCL
AGMAT
LAP3
COQ6
CYP11A1
GK2
MRPS30
PDHX
SARS
MAPK12
OGDHL
MYO19
NDUFV3
FAM65B
PTRH2
FKBP8
B
PCD
PTRH2
FKBP8
GFP-LC3B

Fig. 4. List of the putative positive clones.

Symbol	Full Name	Predicted related pathway	Function	Predicted related disease
FKBP8	FK506-Binding Protein 8	Apoptosis	Bcl2-binding	
PTRH2	peptidyl-tRNA hydrolase 2	Apoptosis	Caspase independent cell death by Trascription factor AES and TLE1	esophagitis
CYB561	cyctochrome B561	Transmembrane electron transfer carrier	specific to catecholamine and neuropeptide secretory vesicles of neuroendocrine tissues	astrocytoma
HMGCL	3-hydroxymethyl -3-Methylglutary l-CoA lyase	Ketone body metabolism	key enzyme in ketogenesis	3-hydroxy-3-methylglutaryl -coenzyme a lyase deficiency
AGMAT	Agmatine Ureohydrolase	Arginine and proline metabolism	Agmantinase	mood disorder
LAP3	leucine aminopeptidase 3	Glutathione metabolism	Release of N-terminal proline from a peptide	bronchial neoplasm
COQ6	coenzyme Q6 monooxygenase	Ubiquinone and other terpenoid -quinone biosynthesis	monooxygenase	nephrotic syndrome
CYP11A1	cytochrome P450, family 11, subfamily A , polypeptide 1	Ovarian steroidogenesis	Catalyzes the side-chain cleavage reaction of cholesterol to pregnenolone	adrenal insufficiency
GK2	Glycerol kinase 2	Glycerolipid metabolism	Key enzyme in the regulation of glycerol uptake and metabolism	hyperglycerolemia
MRPS30	mitochondrial ribosomal protein S30	Mitochondrial ribosome Small Subunit	Structural subunit	breast cancer
PDHX	pyruvate dehydrogenase complex, component X	Pyruvate metabolism	Catalytic subunit E1,E2,E3. require for anchoring E3 to E2	e3-binding protein deficiency
SARS	seryl-tRNA synthetase	Aminoacyl-tRNA biosy nthesis	Catalyzes the attachment of serine to tRNA(Ser).	hyperuricemia
MAPK12	mitogen-activated protein kinase 12	MAPK signaling pathway	Serine/threonine kinase	shigellosis
OGDHL	Oxoglutarate dehydrogenase-like	Citrate cycle	alpha-ketoglutarate dehydrogenase, E1k subunit-like sequence	kidney disease
MYO19	myosin XIX	Mitochondrial motility	ATPase activity	
NDUFV3	NADH dehydrogenase (ubiquinone) flavoprotein 3	Oxidative phosphorylation	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I)	autosomal recessive nonsyndromic deafness
FAM65B	family with sequence similarity 65, member B			prostatitis

Fig. 5. The overexpression of FKBP8 induces colocalization of LC3 family with mitochondria.

(A, B) FKBP8 overexpression induces colocalization of LC3 family and Mito-RFP. Chang liver cells were cotransfected with HA-FKBP8, Mito-RFP and GFP-LC3B (A), GFP-GABARAPL1 or 2 (B). After 24 h, green fluorescence of GFP-LC3 and GFP-GABARAPL1 or 2 and red fluorescence of Mito-RFP were examined under fluorescence microscope.

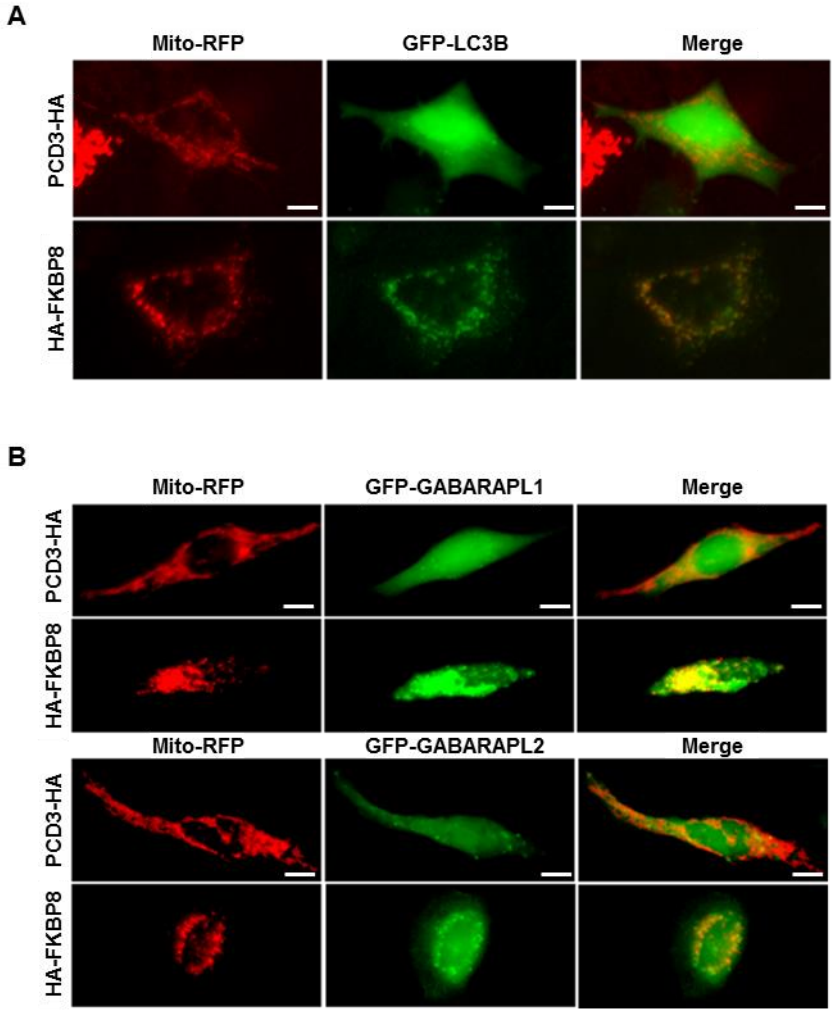

A
Mito-RFP
GFP-LC3B
Merge
PCD3-HA
HA-FKBP8
B
Mito-RFP
GFP-GABARAPL1
Merge
PCD3-HA
HA-FKBP8
Mito-RFP
GFP-GABARAPL2
Merge
PCD3-HA
HA-FKBP8

Fig. 6. The N-terminal region of FKBP8 is required for the recruitment of GFP-LC3B onto mitochondria.

(A) Schematic representation of HA-FKBP8 WT and domain deletion mutants. FKBD: FK506-binding domain, TPR: tetratricopeptide, TM: transmembrane domain. (B) Colocalization of GFP-LC3B and Mito-RFP with HA-FKBP8 deletion mutants. HeLa cells were transiently transfected with HA-FKBP8 deletion mutants, Mito-RFP and GFP-LC3B for 24 h and were then examined under fluorescence microscope.

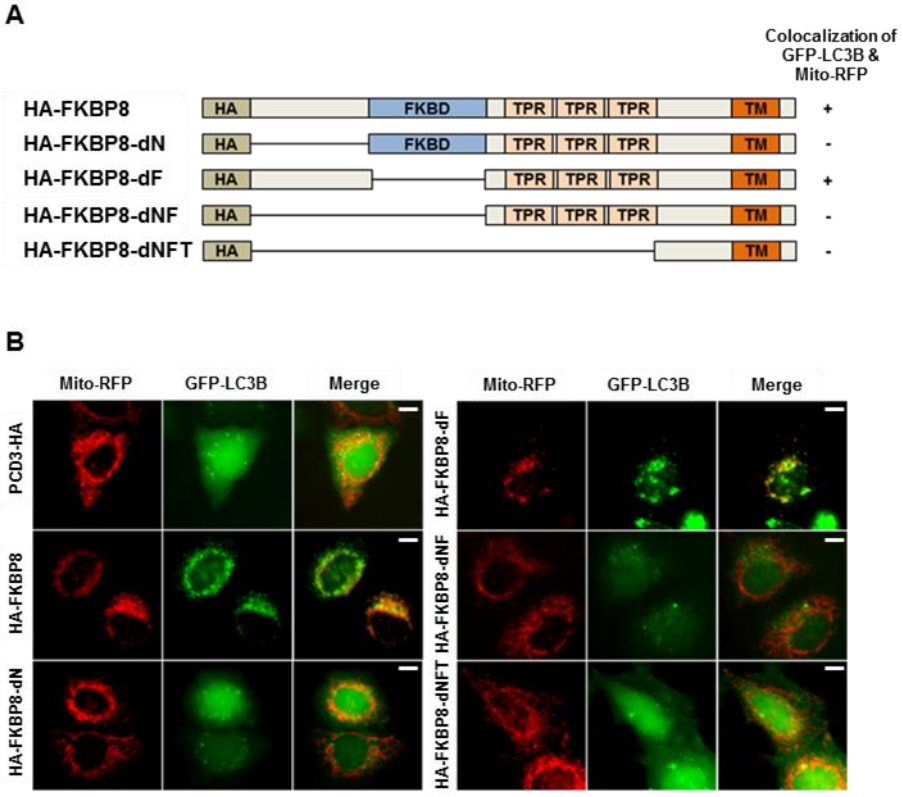

A
Colocalization of GFP-LC3B & Mito-RFP
HA-FKBP8
HA-FKBP8-dN
HA-FKBP8-dF
HA-FKBP8-dNF
HA-FKBP8-dNFT
HA
FKBD
TPR
TM
+
-
B
Mito-RFP
GFP-LC3B
Merge
PCD3-HA
HA-FKBP8
HA-FKBP8-dN
HA-FKBP8-dF
HA-FKBP8-dNF
HA-FKBP8-dNFT

Fig. 7. FKBP8 binds to GFP-LC3B through its N-terminal region.

HEK293T cells were transiently transfected with GFP-LC3B and HA-FKBP8 WT or domain-deletion mutant for 24 h and lyzed with RIPA buffer for 20 min. Cell extracts were then subjected to immunoprecipitation (IP) assay using anti-HA antibody. The immunoprecipitates and whole cell lysates (WCL) were examined by western blotting.

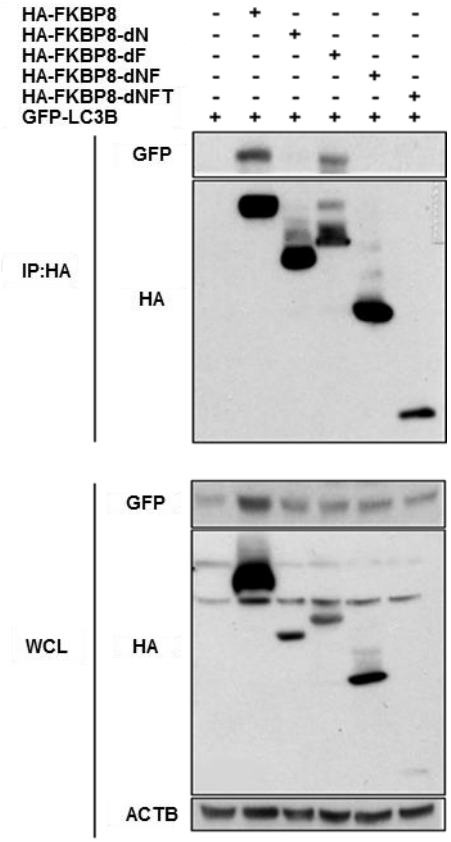

HA-FKBP8 - + - - - -
HA-FKBP8-dN - - + - - -
HA-FKBP8-dF - - - + - -
HA-FKBP8-dNF - - - - + -
HA-FKBP8-dNFT - - - - - +
GFP-LC3B + + + + + +
IP:HA
GFP
HA
WCL
GFP
HA
ACTB

Fig. 8. Prediction of two LIR motives in the N-terminal region of FKBP8.

(A) Comparison of LC3-Interacting-Region (LIR) motives in the LC3-binding proteins and identification of LIR#1 (FEVL) and #2 (WLDI) in FKBP8. (B) Generation of FKBP mutants in the LIR motives. FKBP LIR#1 mutant (F24A-L27A), LIR#2 mutant (W93A-I96A), FKBP LIR#1 & #2 mutant (F24A-L27A, W93A-I96A).

A

ATG32	SGSWQAIQPL
P62	DDDWTHLSSK
NBR	SEDYIIILPE
FUNDC1	DDSYEVLDLT
OPTN	EDSFVEIRMA
FKBP8 LIR #1	LEDFEVLDGV
FKBP8 LIR #2	PEEWLDILGN
LIR motif	**W/Y/F xx I/L**

B

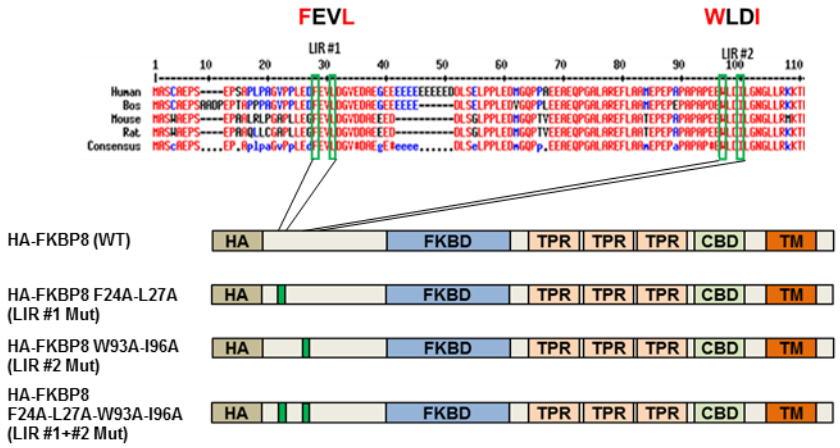


Fig. 9. The LIR#1, but not LIR#2, of FKBP8 is required for the binding to GFP-LC3B and recruiting it onto mitochondria.

(A) Confocal microscope images showing lack of colocalization between GFP-LC3B and HA-FKBP8 LIR#1 mutant. HeLa cells were transfected with GFP-LC3B and either HA-FKBP8 or LIR mutant for 24 h and analyzed under fluorescence microscope. (B) Percentages of GFP-LC3B increased cells with FKBP8 LIR mutants. Bars represent mean ± SD (n = 3). (C) Immunoprecipitation assay showing protein-protein interaction between GFP-LC3B and FKBP8 LIR mutants. HeLa cells were transfected with GFP-LC3B and either HA-FKBP8 or LIR mutant for 24 h, Cell extracts were subjected to immunoprecipitation (IP) assay using anti-HA antibody. The immunoprecipitates and whole cell lysates (WCL) were examined by western blotting.

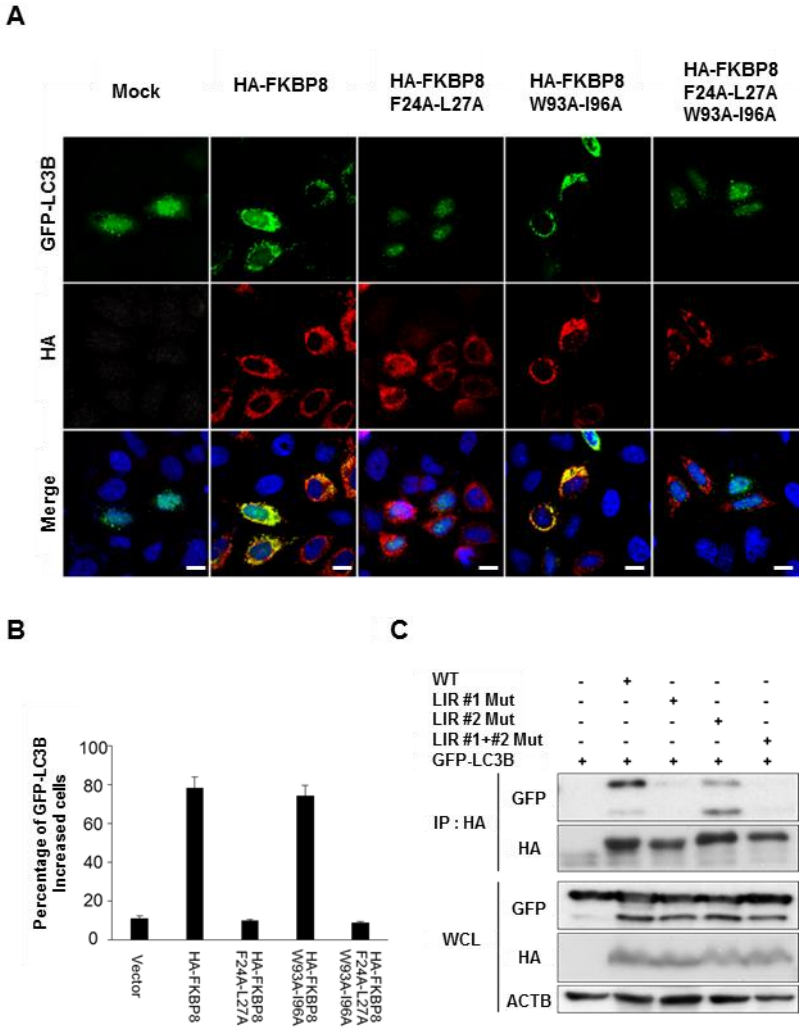

A
Mock
HA-FKBP8
HA-FKBP8 F24A-L27A
HA-FKBP8 W93A-I96A
HA-FKBP8 F24A-L27A W93A-I96A
GFP-LC3B
HA
Merge
B
Percentage of GFP-LC3B Increased cells
100
80
60
40
20
0
Vector
HA-FKBP8
HA-FKBP8 F24A-L27A
HA-FKBP8 W93A-I96A
HA-FKBP8 F24A-L27A W93A-I96A
C
WT
LIR #1 Mut
LIR #2 Mut
LIR #1+#2 Mut
GFP-LC3B
IP : HA
GFP
HA
WCL
GFP
HA
ACTB

Fig. 10. The overexpression of FKBP8 induces mitochondrial fragmentation.

(A) Chang liver and HeLa cells were transfected with either FKBP8 or NIX together with Mito-GFP for 24 h and then examined under fluorescence microscope. (B) Percentage of cells harboring fragmented mitochondria in Fig. (A) were determined and represented as bars with mean ± SD (n = 3).

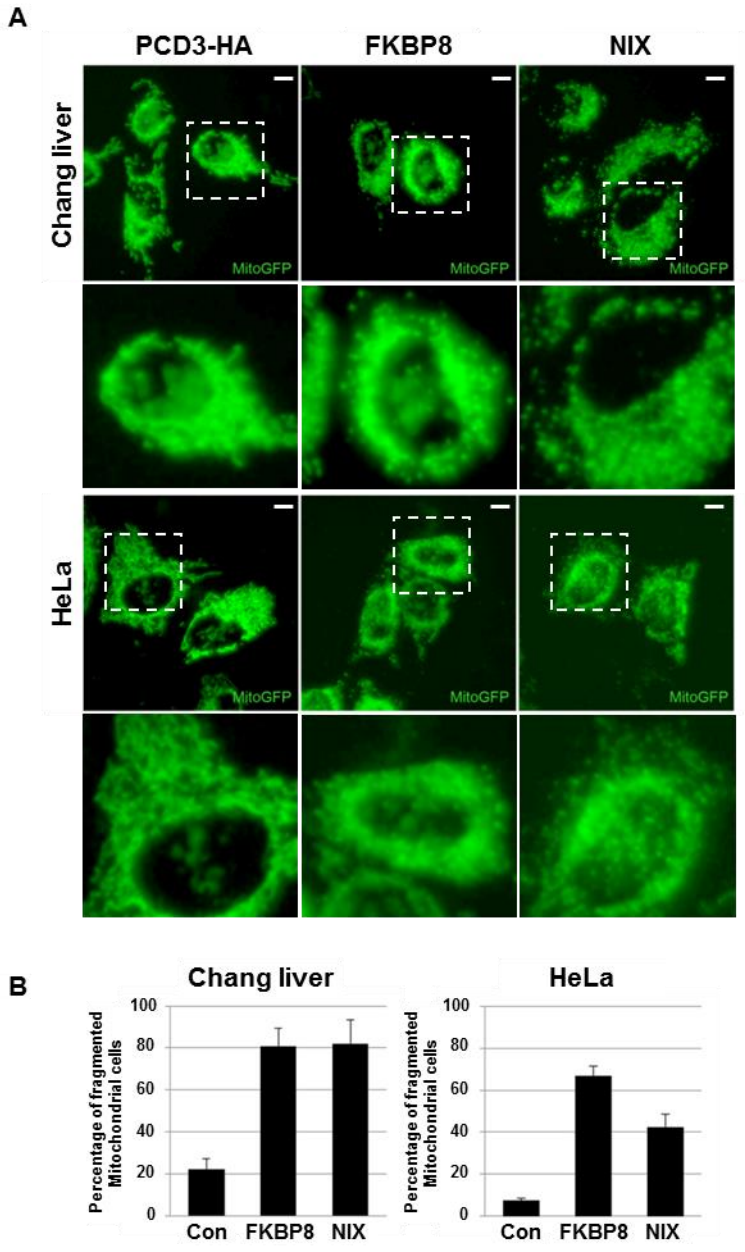
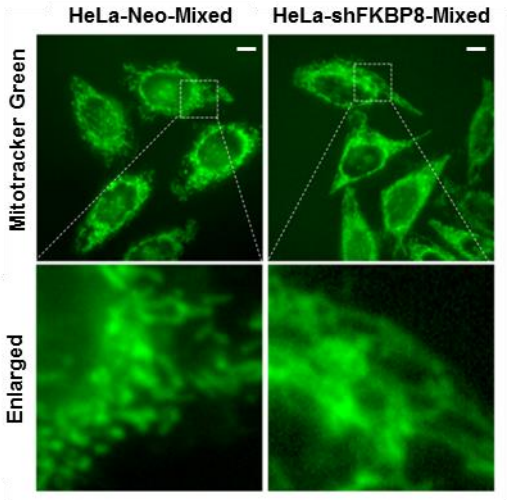

A
PCD3-HA
FKBP8
NIX
Chang liver
HeLa
MitoGFP
B
Chang liver
HeLa
Percentage of fragmented Mitochondrial cells
100
80
60
40
20
0
Con
FKBP8
NIX

Fig. 11. Knockdown of FKBP8 expression increases mitochondrial morphology and mass.

(A) Enlarged morphology of mitochondria in FKBP8 knockdown cells. HeLa cells stably expressing pSUPER-Neo (HeLa-Neo-Mixed) or pFKBP8 shRNA (HeLa-shFKBP8-Mixed) were stained with Mitotracker green (50 μM) for 15 min. The morphology of mitochondria was examined under fluorescence microscope. (B) Western blot assay showing increased mitochondrial proteins. Cell extracts were prepared from HeLa-Neo-Mixed and HeLa-shFKBP8-Mixed cells and analyzed by western blotting using anti-TOMM20 antibody and anti-TIMM23 antibody.

A


HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
Mitotracker Green
Enlarged

B

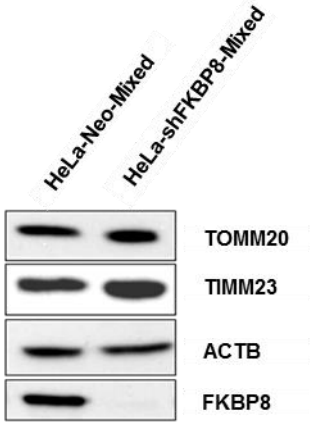

HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
TOMM20
TIMM23
ACTB
FKBP8

Fig. 12. Electron microscopy reveals enlarged and increased numbers of mitochondria in FKBP8 knockdown cells.

(A and B) Electron microscopic images showing enlarged mitochondria in HeLa-shFKBP8-mix cells. (A) Mitochondria in HeLa-Neo-Mixed and HeLa-shFKBP8-Mixed cells were visualized under electron microscope (red arrows). (B) Cellular areas occupied by mitochondria per cells (μM^2) (n = 3) and average size of mitochondrion per μM^2 of HeLa-Neo-Mixed (n = 81) and HeLa-shFKBP8-Mixed (n = 89) cells in the pictures of (A) were measured. Bars represent mean ± SD.

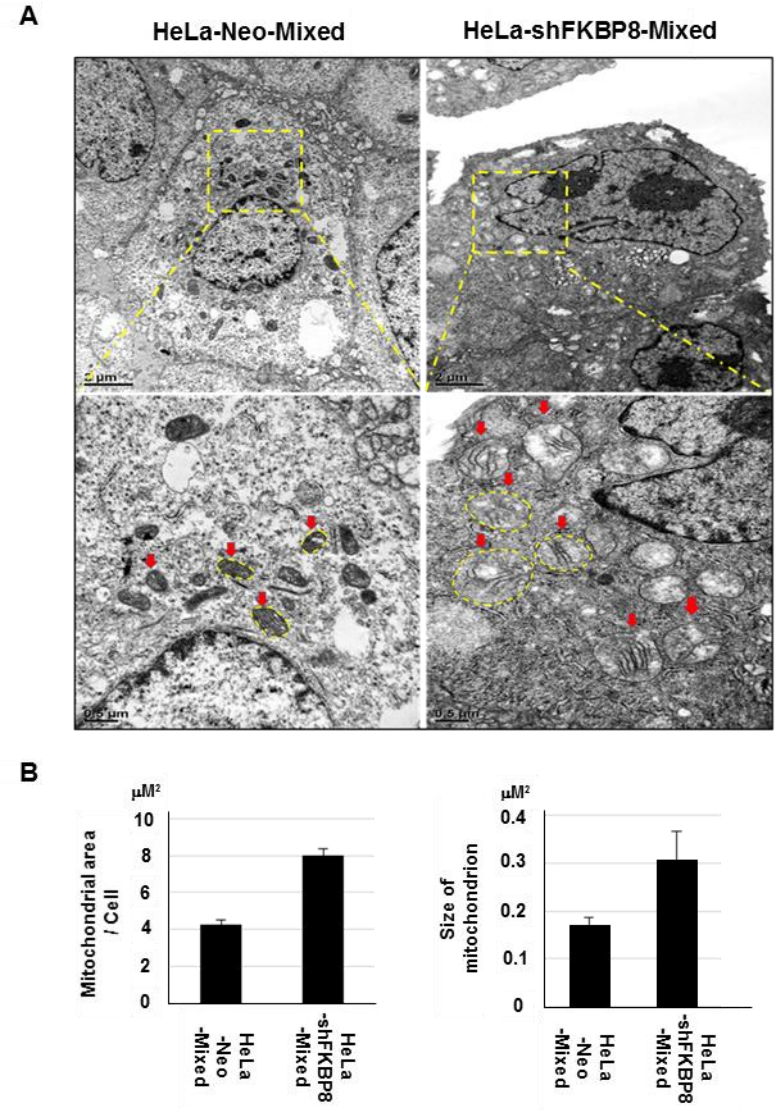

A
HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
2 μm
2 μm
0,5 μm
0,5 μm
B
μM²
Mitochondrial area / Cell
10
8
6
4
2
0
HeLa -Neo -Mixed
HeLa -shFKBP8 -Mixed
μM²
Size of mitochondrion
0.4
0.3
0.2
0.1
0
HeLa -Neo -Mixed
HeLa -shFKBP8 -Mixed

Fig. 13. Mitochondrial fragmentation is induced only by FKBP8 among FKBP8 family.

(A) Subcellular localization and reported function of FKBP family protein. (B) Fragmented mitochondria are observed mainly by FKBP8 overexpression. HeLa cells were transiently transfected with FKBP family together with Mito-GFP and then examined under fluorescence microscope. Bars represent mean ± SD (n = 3).

A

FKBP family	Domains	Subcellular location	Functions
FKBP1A	FKBD	Cytosol	intracelluar signal transduction
FKBP2	FKBD, ER secretion	ER	ER chaperone, membrane cytoskeletal scaffold
FKBP3	FKBD	Nucleus	Interact with nucleic acids, chromatin-related protein and transcription factors
FKBP4	FKBD, TPR	Cytosol, Nucleus, Mitochondrion	Immunoregulatory gene expression in B and T lymphocytes
FKBP5	FKBD, TPR	Cytosol, Nucleus	Cell motility, Glucocorticoid receptor activity
FKBP6	FKBD, TPR	Cytosol, Nucleus	spermatogenic impairment in idiopathic infertile men
FKBP7	FKBD, EF-hand	ER lumen	A similar protein in mouse binds to calcium
FKBP8	FKBD, TPR, Transmembrane	Mitochondrion	Apoptosis, cycstic fibrosis, hepatitis C replication
FKBP9	FKBD, EF-hand	ER	-
FKBP10	FKBD, EF-hand	ER lumen	Osteogenesis
FKBP11	FKBD, predicted integral membrane	membrane	Biomarker for hepatocellular carcinoma
FKBPL	TPR	Cytosol, ER	Breast cancer, estrogen receptor, glucocorticoid receptor signaling

B

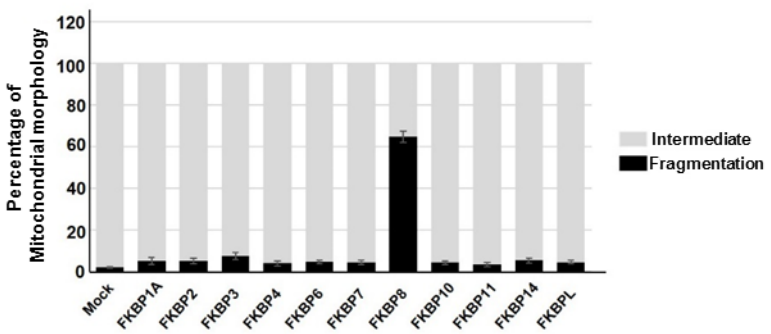


Fig. 14. The N-terminal region and LIR#2 region of FKBP8 is important for FKBP8-induced mitochondrial fragmentation.

(A) Effects of FKBP8 domain-deletion mutants on mitochondrial fragmentation. HeLa cells were transfected with Mito-GFP alone or together with either HA-FKBP8 WT or deletion mutant for 24 h and then examined mitochondrial fragmentation under fluorescence microscope. (C) Effects of FKBP8 LIR mutants on mitochondrial fragmentation. C2C12 cells were transfected with HA-FKBP8 or LIR mutant together with Mito-RFP for 24 h and then examined for mitochondrial fragmentation under fluorescence microscope. (B and D) Percentages of cells harboring fragmented mitochondria. The morphologies of mitochondria in the pictures of Fig. (A and C) were measured and percentages of cells harboring fragmented mitochondria was determined. Bars represent mean ± SD (n = 3).

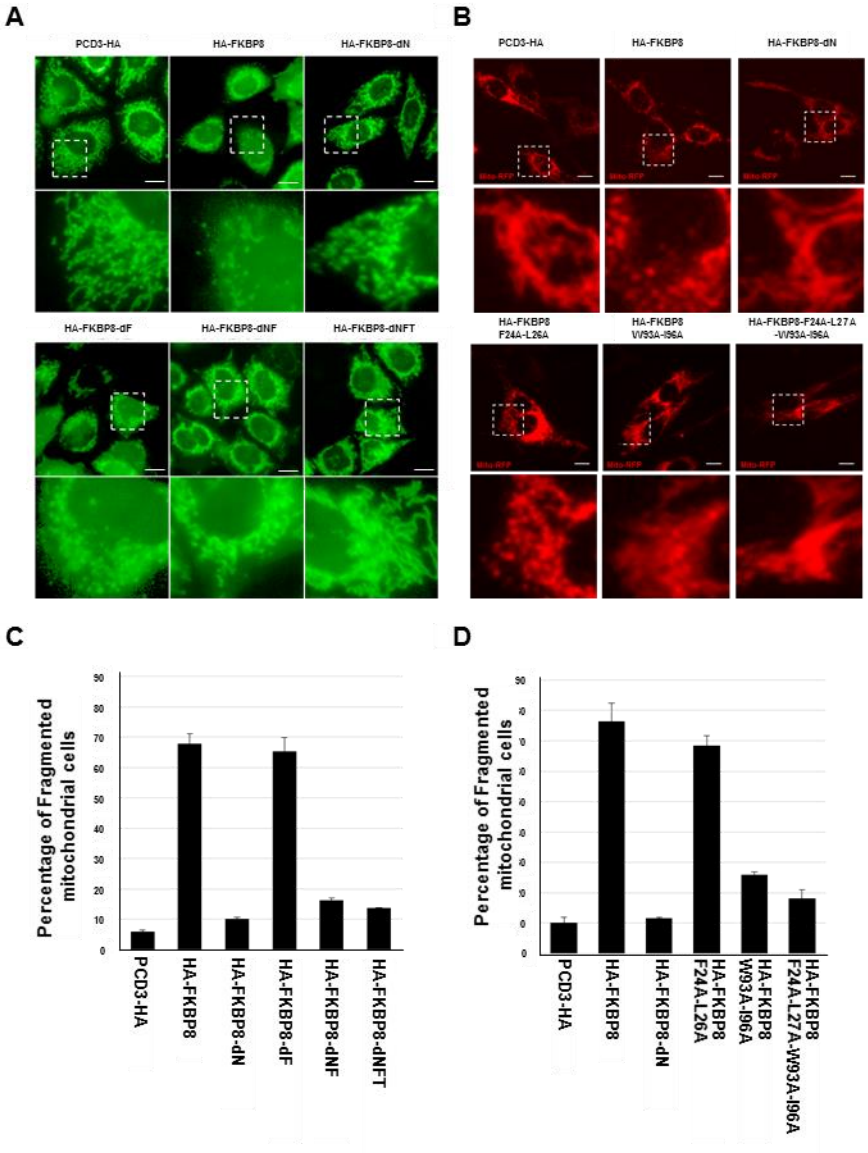

A
PCD3-HA
HA-FKBP8
HA-FKBP8-dN
HA-FKBP8-dF
HA-FKBP8-dNF
HA-FKBP8-dNFT
B
PCD3-HA
HA-FKBP8
HA-FKBP8-dN
HA-FKBP8 F24A-L26A
HA-FKBP8 W93A-I96A
HA-FKBP8-F24A-L27A -W93A-I96A
C
Percentage of Fragmented mitochondrial cells
PCD3-HA
HA-FKBP8
HA-FKBP8-dN
HA-FKBP8-dF
HA-FKBP8-dNF
HA-FKBP8-dNFT
D
Percentage of Fragmented mitochondrial cells
PCD3-HA
HA-FKBP8
HA-FKBP8-dN
HA-FKBP8 F24A-L26A
HA-FKBP8 W93A-I96A
HA-FKBP8 F24A-L27A-W93A-I96A

Fig. 15. Overexpression of FKBP8 induces mitochondrial fragmentation in Drp1 KO MEF.

Overexpressed FKBP8 induces mitochondrial fragmentation without Drp1. (A) WT MEF and Drp1 KO MEF cells were transfected with Mito-RFP and PCD3-HA or HA-FKBP8 for 24 h and examined under fluorescence microscopy. (B) Percentage of cells harboring fragmented mitochondria Fig. (A) was determined.

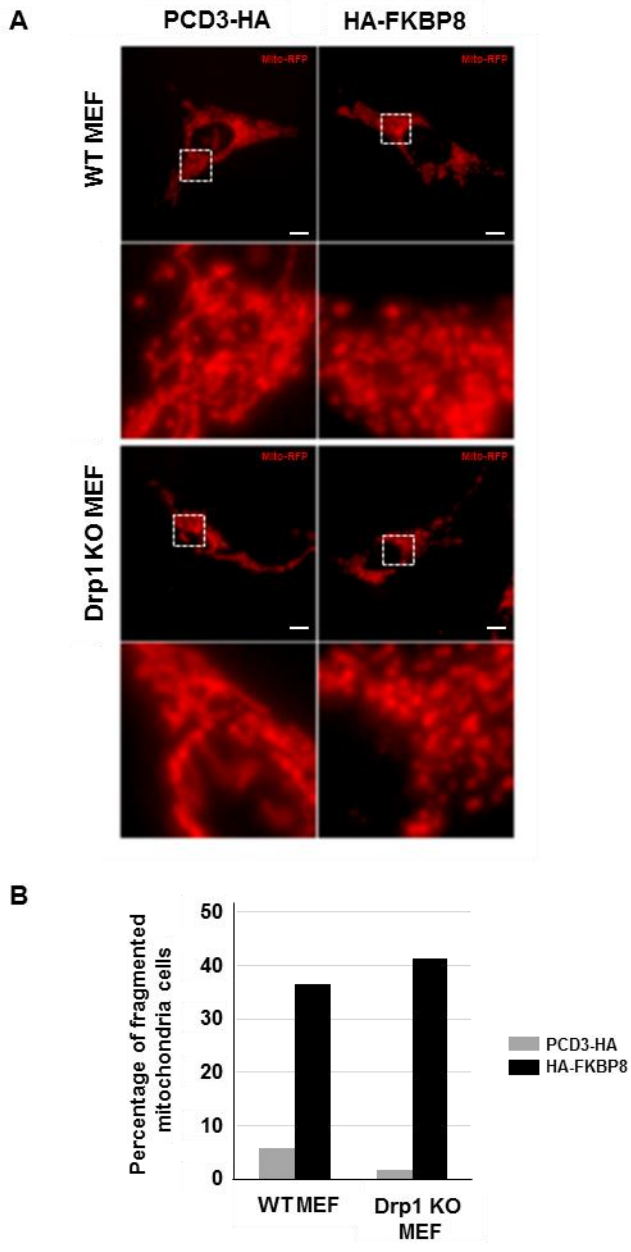

A
PCD3-HA
HA-FKBP8
WT MEF
Drp1 KO MEF
Mito-RFP
B
Percentage of fragmented mitochondria cells
50
40
30
20
10
0
WT MEF
Drp1 KO MEF
PCD3-HA
HA-FKBP8

Fig. 16. Overexpression of FKBP8 induces mitophagy.

HeLa cells stably expressing Mito-Keima (WT, BNIP3/NIX DKO, Drp1 KO, FIP200 KO) are transfected with vector and HA-FKBP8 with pEGFP-C1. After 24 h, Mito-Keima signals in GFP-expressing cells are examined under fluorescence microscopy.

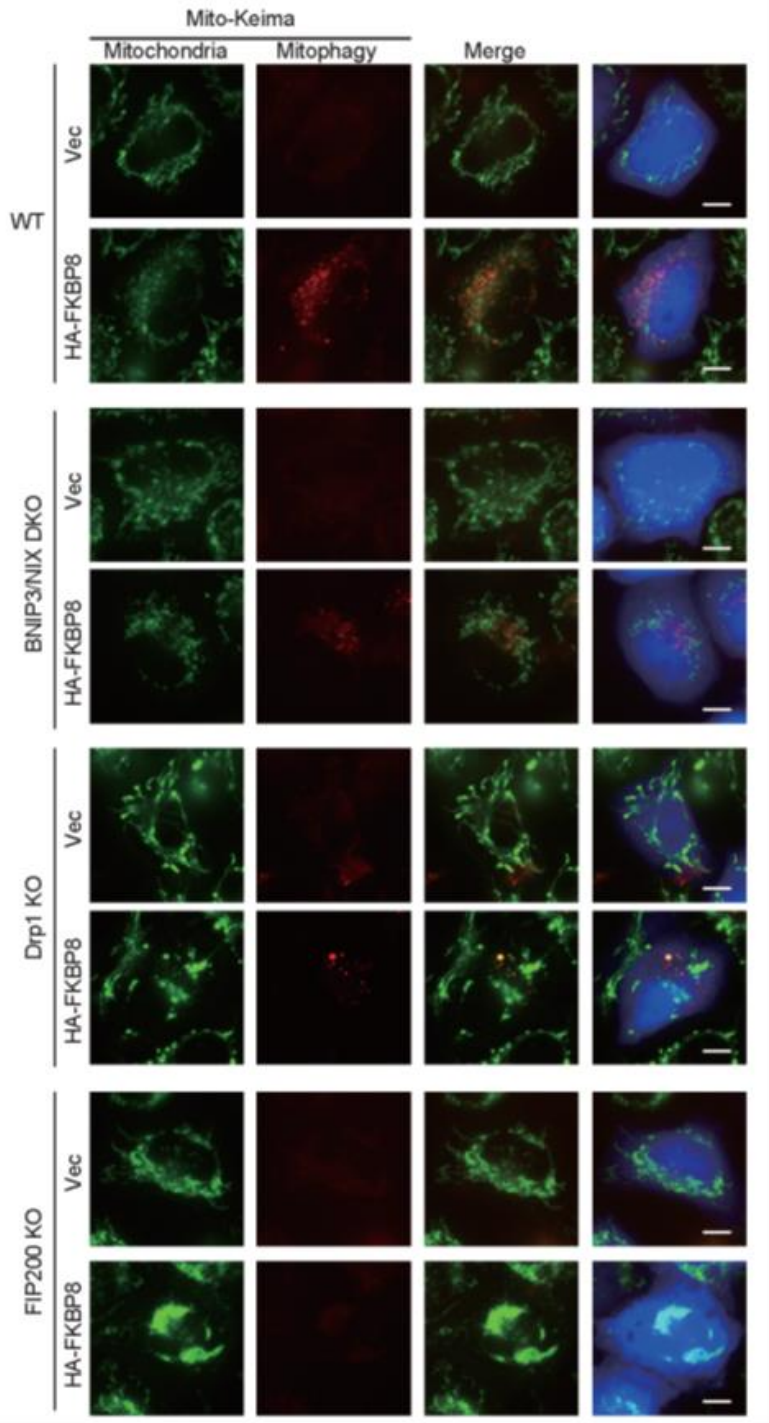

Mito-Keima
Mitochondria
Mitophagy
Merge
WT
Vec
HA-FKBP8
BNIP3/NIX DKO
Vec
HA-FKBP8
Drp1 KO
Vec
HA-FKBP8
FIP200 KO
Vec
HA-FKBP8

Fig. 17. FKBP8 interacts with FIS1 through LIR#2 and TPR domains.

(A) FKBP8 binds to FIS1. HEK293T cells were transiently transfected with FIS1-GFP and HA-FKBP8 WT for 24 h. Cell extracts were then subjected to immunoprecipitation (IP) assay using anti-HA antibody. The immunoprecipitates and whole cell lysates (WCL) were examined by western blotting. (B) Domain mapping of FKBP interacting with FIS1. HEK293T cells were transiently transfected with FIS1-GFP and HA-FKBP8 WT or domain-deletion mutant for 24 h. Cell extracts were then subjected to immunoprecipitation (IP) assay as described in (A).

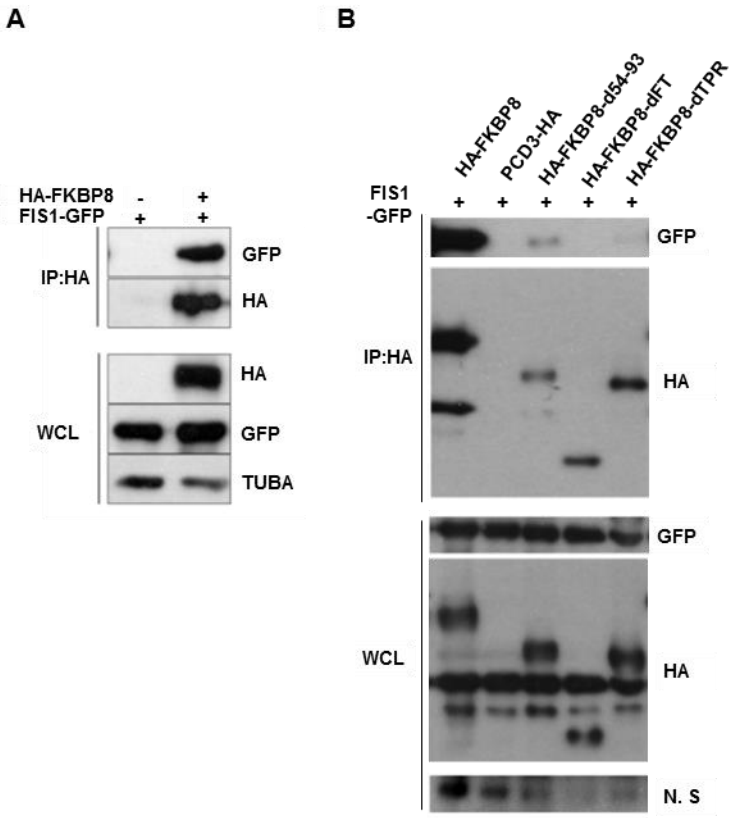

A
HA-FKBP8 - +
FIS1-GFP + +
IP:HA
GFP
HA
WCL
HA
GFP
TUBA
B
HA-FKBP8
PCD3-HA
HA-FKBP8-d54-93
HA-FKBP8-dFT
HA-FKBP8-dTPR
FIS1
-GFP
+ + + + +
GFP
IP:HA
HA
GFP
WCL
HA
N. S

Fig. 18. Knockdown of FKBP8 expression reduces mitochondrial fragmentation under hypoxia.

Inhibition of mitochondrial fragmentation by FKBP8 knockdown during hypoxia. (A and B) Expression levels of FKBP8 during hypoxia. (A) HeLa-Neo-Mixed and HeLa-shFKBP8-Mixed cells are incubated under hypoxia for 24 and 48 h and analyzed by western blotting. (B and C) Morphology of mitochondria under hypoxia. HeLa-Neo-Mixed and HeLa-shFKBP8-Mixed cells are incubated under hypoxia for 48 h. After staining with Mitotracker green (50 μM) for 20 min, cells were analyzed by immunocytochemistry using FKBP8 antibody (red). White arrows and the red arrow indicate the FKBP8 WT cells and the knockdown cell, respectively (B). (D) Mitochondrial morphologies were classified as fragmented, intermediate or tubular structure and their relative ratios were calculated.

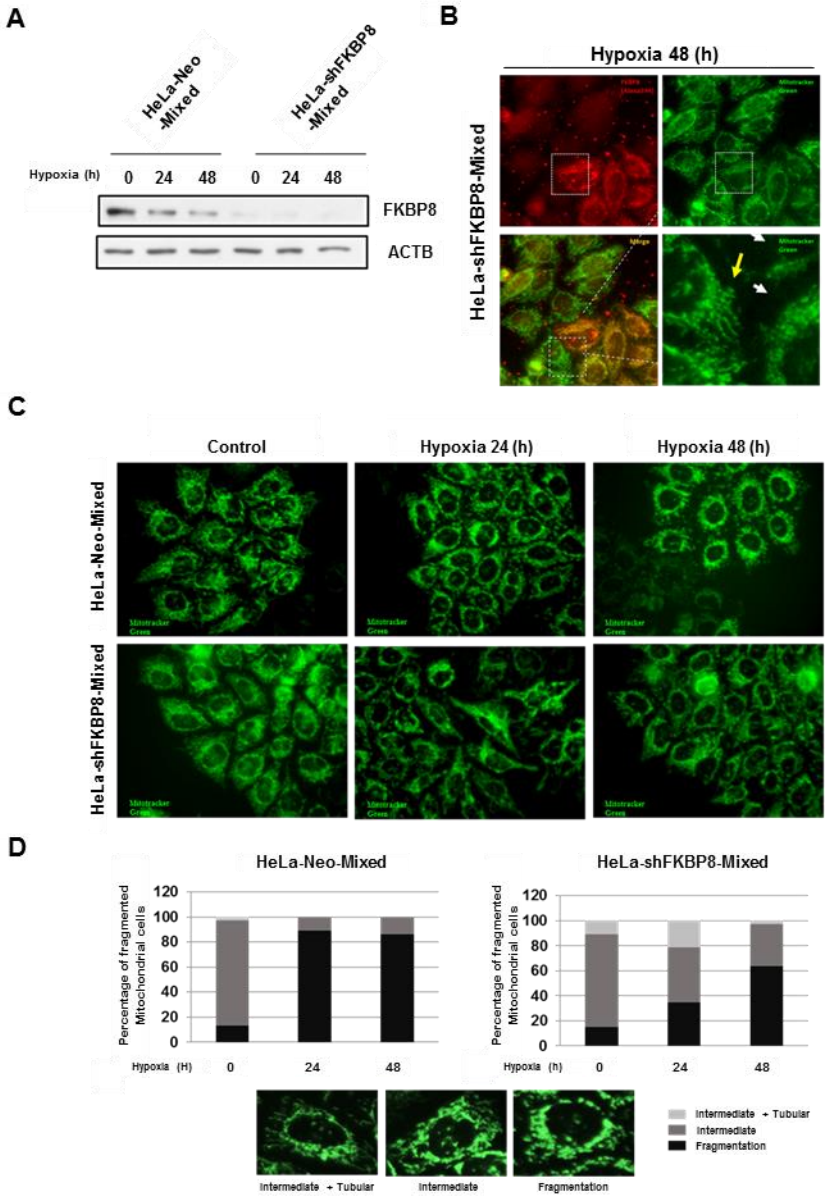

A
HeLa-Neo -Mixed
HeLa-shFKBP8 -Mixed
Hypoxia (h)
0 24 48 0 24 48
FKBP8
ACTB
B
Hypoxia 48 (h)
HeLa-shFKBP8-Mixed
C
Control
Hypoxia 24 (h)
Hypoxia 48 (h)
HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
D
HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
Percentage of fragmented Mitochondrial cells
120
100
80
60
40
20
0
Hypoxia (H)
0
24
48
Intermediate + Tubular
Intermediate
Fragmentation

Fig. 19. FKBP8 affects mitochondrial degradation during hypoxia.

(A and B) Mitochondrial protein levels are regulated by FKBP8 during hypoxia. HeLa-Neo-Mixed and HeLa-shFKBP8-Mixed cells were incubated for 96 h under hypoxia and then analyzed by western blotting (A). HeLa cells were transfected with pcDNA or FKBP8, incubated under hypoxia for 24 h and then analyzed by western blotting. (C) Images of electron microscopic images showing impaired degradation of mitochondria in FKBP8 knockdown cells during hypoxia. HeLa-Neo-Mixed and HeLa-shFKBP8-Mixed cells were exposed to hypoxia for 48 h and then examined by electron microscopy. Red arrow heads indicate mitochondria.

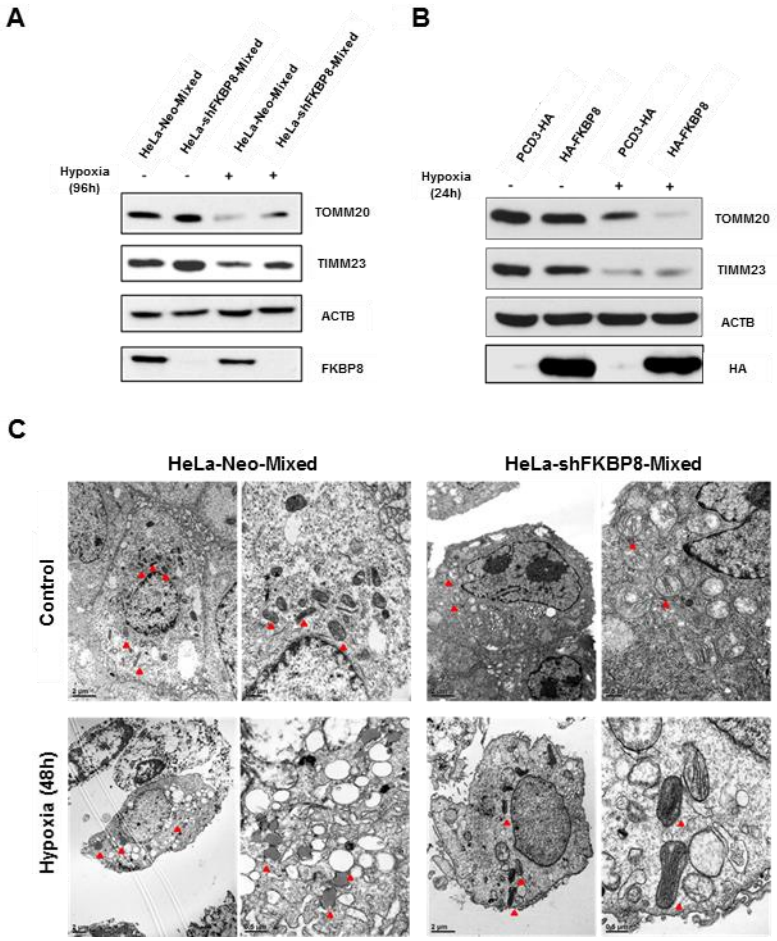
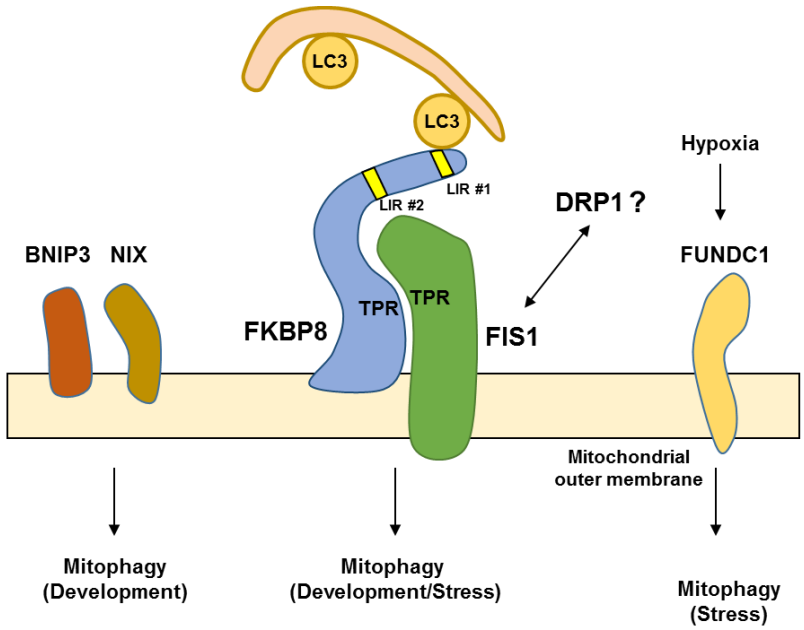

A
HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
Hypoxia (96h)
- - + +
TOMM20
TIMM23
ACTB
FKBP8
B
PCD3-HA
HA-FKBP8
PCD3-HA
HA-FKBP8
Hypoxia (24h)
- - + +
TOMM20
TIMM23
ACTB
HA
C
HeLa-Neo-Mixed
HeLa-shFKBP8-Mixed
Control
Hypoxia (48h)

Fig. 20. The proposed role of FKBP8 in mitophagy and mitochondrial fission.


LC3
LC3
LIR #2
LIR #1
Hypoxia
DRP1 ?
BNIP3
NIX
FKBP8
TPR
TPR
FIS1
FUNDC1
Mitochondrial
outer membrane
Mitophagy
(Development)
Mitophagy
(Development/Stress)
Mitophagy
(Stress)

Discussion

In this study, I have generated and collected about 500 cDNAs which encode mitochondrial proteins in a mammalian expression vector. Based on the literature, the numbers of mitochondrial protein ranges 800 – 900. In order to find a regulatory factor that functions mitophagy, I performed functional screening using cell-based assay employing GFP-LC3. In the assay, I focused on the ability of the mitochondrial protein to recruit GFP-LC3 on the mitochondria upon overexpression based on the assumption that the protein might induce mitochondrial fragmentation for the mitophagy or recruit GFP-LC3 as a receptor of the mitophagy. As I expected, I could successfully isolated several mitochondrial proteins that affected the recruitment of GFP-LC3. This type of GOF screening can have an advantage over LOF screening to isolate a regulator in a signaling that is not operating in the assay and thus cannot be uncovered by LOF screening (Han et al., 2014; Jung et al., 2016).

Among the putative positive clones, I decided to characterize the role of FKBP8 in the mitophagy. The reported role of FKBP8 in the mitochondria is not clear yet. Though FKBP8 has peptidylprolyl

isomerase domain, this enzymatic activity is not likely functional (Edlich et al., 2005) and thus is not related to mitophagic function. FKBP8 has also a FK506-binding domain but this function in mitophagy is nothing to do with mitophagy because deletion of the domain did not affect mitophagy. Moreover, previous report showed that FKBP8 escapes from the mitochondria to ER during CCCP-induced mitophagy and that this escape from ER is dependent on Parkin. However, I hardly observed that FKBP8 escaped from the mitochondria in the presence or absence of Parkin following CCCP treatment or exposure to hypoxic condition (data not shown).

Unlike other LIR-containing proteins, FKBP8 has two LIR motives in the N-terminus. Most LC3-binding proteins have one LIR and LIRs are known to be involved in the binding to LC3. TBC1D5 has been reported to have two LIR motives, one responsible for interaction with LC3 and the other LIR required for attachment to the retromer (Popovic et al., 2012). Similarly, two LIR motifs of FKBP8 are believed to have different role. As shown in TBC1D5, the first LIR of FKBP8 binds to LC3 and the second LIR plays a distinct role in mitochondria dynamics. Mutation in the second LIR attenuated its ability to induce mitochondrial fragmentation following ectopic expression.

Then, an important question how FKBP8 mediates mitochondria

dynamics and fragmentation remains to be addressed. Similar activity to affect mitochondria dynamics and fragmentation is also detected in FUNDC1 and NIX. Recently, it was reported that FUNDC1 recruits DRP1 through calnexin (Wu et al., 2016). However, the necessity of DRP1 in this function of FUNDC1 is not known yet. I also found that FKBP8 binds to DRP1 with immunoprecipitation assays but ectopic expression of FKBP8 still induced mitochondrial fragmentation in DRP1 KO MEF cells (data not shown), indicating that DRP1 is not a downstream main mediator of FKBP8 in mitochondrial fragmentation.

I then tested whether FKPB8 interacted with other mitochondrial fusion/fission factors. Among them, I found that FKBP8 bound to FIS1, a protein involved in mitochondrial fission (Mozdy, McCaffery, & Shaw, 2000), in the immunoprecipitation and this interaction required TPR domain of FKBP8. From careful analysis using FKBP8 deletion mutants, I noticed that compared to full-length FKBP8, TPR domain deletion mutant also exhibited reduced ability to induce mitochondrial fragmentation (data not shown). While an assay to test a role of FIS1 in FKBP8-mediated mitochondrial fragmentation remains to be addressed, FIS1 appears to be involved in the mitochondrial fission or mitophagy as a binding partner. FIS1 interacts with DRP1, MIEF1, and MiD51 for mitochondrial fission and LC3 accumulates in cells without FIS1

(Yamano, Fogel, Wang, van der Bliek, & Youle, 2014). Further detail analysis for the interaction of FBKBP8 with these factors needs to be investigated. From LC/MS analysis using FKBP8 WT and FKBP8-d54-93, and OPA1, which is fusion regulator of mitochondrial inner membrane, shows different value. Electron microscopic figure of HeLa-shFKBP8-Mixed cells shows that the cristae of mitochondria are decreased and similar to mitochondria with OPA1 KO cells (Patten et al., 2014). Further studies with OPA1 seem to be needed.

NIX and BNIP3 are important regulator of mitophagy in development and mitochondrial dynamics (Glick et al., 2012). BNIP3 and NIX localize to the outer mitochondrial membrane, where it functions in mitophagy and mitochondrial dynamics. However, the observation that FKBP8 induces mitophagy in NIX/BNIP3 double knockout (DKO) HeLa cells suggest that FKBP8-mediated mitochondrial dynamics or mitophagy is distinct from NIX and BNIP3. Interestingly, FKBP8 may play a role in mitophagy under hypoxia, implicating that FKBP8 may also be involved in stress-induced mitophagy. Especially, FKBP8 KO mice are lethal and FKBP8 KO mice generated with Genetrap method exhibit neural tube defects in the back (Wong et al., 2008). Thus, I speculate that failure of mitochondrial quality control through mitochondrial dynamics and mitophagy may cause neural defect in

FKBP8 KO mice, which will be examined.

In summary, my study suggests that FKBP8 contributes to mitophagy as a bifunctional protein for mitochondrial quality control through the interaction with LC3 and to mitochondrial fission through the interaction with FIS1 (Fig. 20).

MATERIALS AND METHODS

DNA construction

The GFP-LC3 expression construct was described elsewhere (Kabeya et al., 2000). FKBP8 shRNA constructs were generated in pSUPER-neo (OligoEngine, VEC-PBS-0004) using the following target sequence for human FKBP8 (5'-AGT GGA CAT GAC GTT CGA GGA-3') .Human FKBP8 (NM_012181.3) was amplified by PCR and ligated into pcDNA3-HA The following nucleotide sequences were used as the primers: FKBP8-Not I-Sense (5′-AGA ATG CGG CCG CTA ATG GCA TCG TG-3′), FKBP8-Xba I-Antisense (5'-CTA GTC TAG ATC AGT TCC TGG CAG CG-3'), FKBP8 dN-Not I-Sense(5'-ATA AGA ATG CGG CCG CTA AAG AAG ACG CTG GTC CCA GG-3'), FKBP8 dNdFKBD-Not I-Sense(5'-ATA AGA ATG CGG CCG CTA ACG GCT GTG GAC GGG CCT G-3'), FKBP8 dNdFKBDdTPR-Not I-Sense(5'-ATA AGA ATG CGG CCG CTA ACG ATC CAC GCA GAG CTC T-3'), FKBP8 dFKBD(113AA)-Bgl II-Antisense(5'-GAA GAT CTT CTG GCG GCC CTG GGA CCA GCG-3'), FKBP8 dFKBD(202AA)-Bgl II-Sense(5'-GAA GAT CTT ACG GCT GTG GAC GGG CCT G-3'), and

other deletion mutants were generated by PCR without gap using HA-FKBP8 plasmid.

Cell culture, DNA transfection and generation of stable cell line

All cells were cultured in Dulbecco's modified Eagle medium (Hyclone, SH30243.01) supplemented with 10 % fetal bovine serum (Hyclone, SH30919.03), 100 Units/ml gentamycin. HEK293T cells were transfected using PEI reagent (sigma) for 24 h, typically, 2×10^5 cells per well in 6-well culture plates were transfected with appropriate plasmids. And Chang liver, HeLa and MEFs were transfected using Polyfect reagent (Quiagen) according to the manufacturer's protocol. For generation of HeLa-shFKBP8-Mixed stable cell line, HeLa cells transfected with the plasmid for 24 h and then incubated with G418 for additional 14 days. Drp1 knockout MEFs was kind gifts from K Mihara (Kyushu University, Fukuoka, Japan). KO cells used in Fig. 16 are described elsewhere (Yamashita et al., 2016).

Immunocytochemistry

HeLa cells grown on coverslips in 12-well plates were fixed in 4%

paraformaldehyde for 5 min, permeabilized with 0.1% triton x-100 in PBS for 5 min, and blocked with 1% BSA for 1 h. After blocking, the fixed cells were incubated with antibodies at 1:100 to 1:250 dilution ratio, followed by incubation with Alexa Fluor secondary antibodies (Molecular probes, A11005, A11001, A11008 and A11012) and Hoechst 33258. Samples were visualized under a confocal fluorescence microscope (Carl Zeiss, LSM700, Carl-Zeiss-Promenade 10, 07745, Jena Germany).

Western blot and antibodies

The following antibodies were used : HA, GFP (sc-8334), TUBA/tubulin-α (sc-23948), ACTB (sc-47778), TOMM20 (sc-17764), TIMM23 (BD Bioscience, 611222), LC3 (Novus NB100-2220) and FKBP8 (R&D system, MAB3580). Western blot analysis were performed using standard techniques. Cells were lysed with SDS sample buffer (10% glycerol, 2% SDS, 5% β-mercaptoethanol, pH 6.8, 50mM Tris-Cl, pH 6.8).

Immunoprecipitation, western blot, and antibodies

Cells were lysed in Modified RIPA buffer (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Triton X-100) and clarified by centrifugation. Cell lysates were dichotomized for immunoprecipitation and whole cell lysate quantification. For immunoprecipitaion, HA antibody was added overnight at 4°C and pulled down by protein G Sepharose beads (GE Healthcare, 17-0618-01) at 4°C for 3 h. Beads are washed 5 times with Modified RIPA buffer for 5min at 4°C. Samples were boiled for 15 min in SDS sample buffer, separated by SDS-PAGE and transferred onto PVDF membrane. After blocking with 3% BSA in TBST, the membrane was incubated with the indicated antibodies.

Transmission electron microscope analysis

Cells were fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), followed by 1% OsO4. Cells were further dehydrated with a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate for observation under a JEM1010 transmission electron microscope (JEOL).

Statistics

All experiments were performed in triplicate parallel instances and repeated at least three times. Statistical analyses were carried out using the Microsoft Office 2013 Excel software package (Microsoft Corporation).

REFERENCES

Archer, S. L. (2013). Mitochondrial dynamics-mitochondrial fission and fusion in human diseases. *N Engl J Med, 369*(23), 2236-2251. doi:10.1056/NEJMra1215233

Ballard, J. W., & Youngson, N. A. (2015). Review: can diet influence the selective advantage of mitochondrial DNA haplotypes? *Biosci Rep, 35*(6). doi:10.1042/BSR20150232

Bratic, I., & Trifunovic, A. (2010). Mitochondrial energy metabolism and ageing. *Biochim Biophys Acta, 1797*(6-7), 961-967. doi:10.1016/j.bbabio.2010.01.004

Chen, Y. G., Liu, F., & Massague, J. (1997). Mechanism of TGFbeta receptor inhibition by FKBP12. *EMBO J, 16*(13), 3866-3876. doi:10.1093/emboj/16.13.3866

Ding, W. X., & Yin, X. M. (2012). Mitophagy: mechanisms, pathophysiological roles, and analysis. *Biol Chem, 393*(7), 547-564.

doi:10.1515/hsz-2012-0119

Duchen, M. R. (2004). Mitochondria in health and disease: perspectives on a new mitochondrial biology. *Mol Aspects Med, 25*(4), 365-451. doi:10.1016/j.mam.2004.03.001

Edlich, F., Weiwad, M., Erdmann, F., Fanghanel, J., Jarczowski, F., Rahfeld, J. U., & Fischer, G. (2005). Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin. *EMBO J, 24*(14), 2688-2699. doi:10.1038/sj.emboj.7600739

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol, 221*(1), 3-12. doi:10.1002/path.2697

Glick, D., Zhang, W., Beaton, M., Marsboom, G., Gruber, M., Simon, M. C. Macleod, K. F. (2012). BNip3 regulates mitochondrial function and lipid metabolism in the liver. *Mol Cell Biol, 32*(13), 2570-2584. doi:10.1128/MCB.00167-12

Han, J., Jung, S., Jang, J., Kam, T. I., Choi, H., Kim, B. J., . . . Jung, Y. K. (2014). OCIAD2 activates gamma-secretase to enhance amyloid beta production by interacting with nicastrin. *Cell Mol Life Sci, 71*(13),

2561-2576. doi:10.1007/s00018-013-1515-x

Hara, T., Takamura, A., Kishi, C., Iemura, S., Natsume, T., Guan, J. L., & Mizushima, N. (2008). FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol, 181*(3), 497-510. doi:10.1083/jcb.200712064

Haupt, K., Jahreis, G., Linnert, M., Maestre-Martinez, M., Malesevic, M., Pechstein, A., . . . Lucke, C. (2012). The FKBP38 catalytic domain binds to Bcl-2 via a charge-sensitive loop. *J Biol Chem, 287*(23), 19665-19673. doi:10.1074/jbc.M111.317214

Hutt, D. M., Roth, D. M., Chalfant, M. A., Youker, R. T., Matteson, J., Brodsky, J. L., & Balch, W. E. (2012). FK506 binding protein 8 peptidylprolyl isomerase activity manages a late stage of cystic fibrosis transmembrane conductance regulator (CFTR) folding and stability. *J Biol Chem, 287*(26), 21914-21925. doi:10.1074/jbc.M112.339788

Ishihara, N., Nomura, M., Jofuku, A., Kato, H., Suzuki, S. O., Masuda, K., . . . Mihara, K. (2009). Mitochondrial fission factor Drp1 is

essential for embryonic development and synapse formation in mice. *Nat Cell Biol, 11*(8), 958-966. doi:10.1038/ncb1907

Jin, Y. J., & Burakoff, S. J. (1993). The 25-kDa FK506-binding protein is localized in the nucleus and associates with casein kinase II and nucleolin. *Proc Natl Acad Sci U S A, 90*(16), 7769-7773.

Jung, S., Nah, J., Han, J., Choi, S. G., Kim, H., Park, J., . . . Jung, Y. K. (2016). Dual-specificity phosphatase 26 (DUSP26) stimulates Abeta42 generation by promoting amyloid precursor protein axonal transport during hypoxia. *J Neurochem, 137*(5), 770-781. doi:10.1111/jnc.13597

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., . . . Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J, 19*(21), 5720-5728. doi:10.1093/emboj/19.21.5720

Lam, E., Martin, M., & Wiederrecht, G. (1995). Isolation of a cDNA encoding a novel human FK506-binding protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene, 160*(2), 297-

302.

Lin, I. Y., Yen, C. H., Liao, Y. J., Lin, S. E., Ma, H. P., Chan, Y. J., & Chen, Y. M. (2013). Identification of FKBP11 as a biomarker for hepatocellular carcinoma. *Anticancer Res, 33*(6), 2763-2769.

Mamane, Y., Sharma, S., Petropoulos, L., Lin, R., & Hiscott, J. (2000). Posttranslational regulation of IRF-4 activity by the immunophilin FKBP52. *Immunity, 12*(2), 129-140.

McKeen, H. D., Byrne, C., Jithesh, P. V., Donley, C., Valentine, A., Yakkundi, A., . . . Robson, T. (2010). FKBPL regulates estrogen receptor signaling and determines response to endocrine therapy. *Cancer Res, 70*(3), 1090-1100. doi:10.1158/0008-5472.CAN-09-2515

McKeen, H. D., McAlpine, K., Valentine, A., Quinn, D. J., McClelland, K., Byrne, C., . . . Robson, T. (2008). A novel FK506-like binding protein interacts with the glucocorticoid receptor and regulates steroid receptor signaling. *Endocrinology, 149*(11), 5724-5734. doi:10.1210/en.2008-0168

Mozdy, A. D., McCaffery, J. M., & Shaw, J. M. (2000). Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J Cell Biol, 151*(2), 367-380.

Nelson, L., McKeen, H. D., Marshall, A., Mulrane, L., Starczynski, J., Storr, S. J., . . . Robson, T. (2015). FKBPL: a marker of good prognosis in breast cancer. *Oncotarget, 6*(14), 12209-12223. doi:10.18632/oncotarget.3528

Ni, H. M., Williams, J. A., & Ding, W. X. (2015). Mitochondrial dynamics and mitochondrial quality control. *Redox Biol, 4*, 6-13. doi:10.1016/j.redox.2014.11.006

Nigam, S. K., Jin, Y. J., Jin, M. J., Bush, K. T., Bierer, B. E., & Burakoff, S. J. (1993). Localization of the FK506-binding protein, FKBP 13, to the lumen of the endoplasmic reticulum. *Biochem J, 294 (Pt 2)*, 511-515.

Novak, I., Kirkin, V., McEwan, D. G., Zhang, J., Wild, P., Rozenknop, A., . . . Dikic, I. (2010). Nix is a selective autophagy receptor for

mitochondrial clearance. *EMBO Rep, 11*(1), 45-51. doi:10.1038/embor.2009.256

Nunnari, J., & Suomalainen, A. (2012). Mitochondria: in sickness and in health. *Cell, 148*(6), 1145-1159. doi:10.1016/j.cell.2012.02.035

Okamoto, T., Omori, H., Kaname, Y., Abe, T., Nishimura, Y., Suzuki, T., . . . Matsuura, Y. (2008). A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. *J Virol, 82*(7), 3480-3489. doi:10.1128/JVI.02253-07

Patten, D. A., Wong, J., Khacho, M., Soubannier, V., Mailloux, R. J., Pilon-Larose, K., . . . Slack, R. S. (2014). OPA1-dependent cristae modulation is essential for cellular adaptation to metabolic demand. *EMBO J, 33*(22), 2676-2691. doi:10.15252/embj.201488349

Pedersen, K. M., Finsen, B., Celis, J. E., & Jensen, N. A. (1999). muFKBP38: a novel murine immunophilin homolog differentially expressed in Schwannoma cells and central nervous system neurons in vivo. *Electrophoresis, 20*(2), 249-255. doi:10.1002/(SICI)1522-2683(19990201)20:2<249::AID-ELPS249>3.0.CO;2-F

Popovic, D., Akutsu, M., Novak, I., Harper, J. W., Behrends, C., & Dikic, I. (2012). Rab GTPase-activating proteins in autophagy: regulation of endocytic and autophagy pathways by direct binding to human ATG8 modifiers. *Mol Cell Biol, 32*(9), 1733-1744. doi:10.1128/MCB.06717-11

Reddy, P. H., Reddy, T. P., Manczak, M., Calkins, M. J., Shirendeb, U., & Mao, P. (2011). Dynamin-related protein 1 and mitochondrial fragmentation in neurodegenerative diseases. *Brain Res Rev, 67*(1-2), 103-118. doi:10.1016/j.brainresrev.2010.11.004

Saita, S., Shirane, M., & Nakayama, K. I. (2013). Selective escape of proteins from the mitochondria during mitophagy. *Nat Commun, 4*, 1410. doi:10.1038/ncomms2400

Schwarze, U., Cundy, T., Pyott, S. M., Christiansen, H. E., Hegde, M. R., Bank, R. A., . . . Byers, P. H. (2013). Mutations in FKBP10, which result in Bruck syndrome and recessive forms of osteogenesis imperfecta, inhibit the hydroxylation of telopeptide lysines in bone collagen. *Hum Mol Genet, 22*(1), 1-17. doi:10.1093/hmg/dds371

Shadidy, M., Caubit, X., Olsen, R., Seternes, O. M., Moens, U., & Krauss, S. (1999). Biochemical analysis of mouse FKBP60, a novel member of the FKPB family. *Biochim Biophys Acta, 1446*(3), 295-307.

Shirane, M., & Nakayama, K. I. (2003). Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol, 5*(1), 28-37. doi:10.1038/ncb894

Stavru, F., Palmer, A. E., Wang, C., Youle, R. J., & Cossart, P. (2013). Atypical mitochondrial fission upon bacterial infection. *Proc Natl Acad Sci U S A, 110*(40), 16003-16008. doi:10.1073/pnas.1315784110

Takaoka, M., Ito, S., Miki, Y., & Nakanishi, A. (2017). FKBP51 regulates cell motility and invasion via RhoA signaling. *Cancer Sci, 108*(3), 380-389. doi:10.1111/cas.13153

Thomson, A. W., Bonham, C. A., & Zeevi, A. (1995). Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. *Ther Drug Monit, 17*(6), 584-591.

Toneatto, J., Guber, S., Charo, N. L., Susperreguy, S., Schwartz, J., Galigniana, M. D., & Piwien-Pilipuk, G. (2013). Dynamic mitochondrial-nuclear redistribution of the immunophilin FKBP51 is regulated by the PKA signaling pathway to control gene expression during adipocyte differentiation. *J Cell Sci*, *126*(Pt 23), 5357-5368. doi:10.1242/jcs.125799

Wild, P., McEwan, D. G., & Dikic, I. (2014). The LC3 interactome at a glance. *J Cell Sci*, *127*(Pt 1), 3-9. doi:10.1242/jcs.140426

Wong, R. L., Wlodarczyk, B. J., Min, K. S., Scott, M. L., Kartiko, S., Yu, W., . . . Finnell, R. H. (2008). Mouse Fkbp8 activity is required to inhibit cell death and establish dorso-ventral patterning in the posterior neural tube. *Hum Mol Genet*, *17*(4), 587-601. doi:10.1093/hmg/ddm333

Wu, W., Li, W., Chen, H., Jiang, L., Zhu, R., & Feng, D. (2016). FUNDC1 is a novel mitochondrial-associated-membrane (MAM) protein required for hypoxia-induced mitochondrial fission and mitophagy. *Autophagy*, *12*(9), 1675-1676. doi:10.1080/15548627.2016.1193656

Yamano, K., Fogel, A. I., Wang, C., van der Bliek, A. M., & Youle, R. J. (2014). Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy. *Elife, 3*, e01612. doi:10.7554/eLife.01612

Yamashita, S. I., Jin, X., Furukawa, K., Hamasaki, M., Nezu, A., Otera, H., . . . Kanki, T. (2016). Mitochondrial division occurs concurrently with autophagosome formation but independently of Drp1 during mitophagy. *J Cell Biol, 215*(5), 649-665. doi:10.1083/jcb.201605093

Youle, R. J., & Narendra, D. P. (2011). Mechanisms of mitophagy. *Nat Rev Mol Cell Biol, 12*(1), 9-14. doi:10.1038/nrm3028

Zhang, W., Zhang, S., Xiao, C., Yang, Y., & Zhoucun, A. (2007). Mutation screening of the FKBP6 gene and its association study with spermatogenic impairment in idiopathic infertile men. *Reproduction, 133*(2), 511-516. doi:10.1530/REP-06-0125

Zhang, X., Wang, Y., Li, H., Zhang, W., Wu, D., & Mi, H. (2004). The mouse FKBP23 binds to BiP in ER and the binding of C-terminal domain is interrelated with Ca2+ concentration. *FEBS Lett, 559*(1-3),

57-60. doi:10.1016/S0014-5793(04)00024-9

Zhu, Y., Massen, S., Terenzio, M., Lang, V., Chen-Lindner, S., Eils, R., . . . Brady, N. R. (2013). Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis. *J Biol Chem, 288*(2), 1099-1113. doi:10.1074/jbc.M112.399345

국 문 초 록

미토콘드리아는 환경적 스트레스나 대사적 스트레스에 대응해 정상적인 기능을 유지하기 위하여 끊임없이 분열과 융합을 일으켜 형태를 바꾼다. 미토콘드리아의 동역학적 분열과 융합이 제대로 기능하지 못 하거나 미토콘드리아의 분해에 이상이 생기면, 정상적인 미토콘드리아의 질을 유지하는데 실패하며, 이는 사람에서의 여러 질병과 연관되어 있다고 알려져 있다. 따라서, 미토콘드리아의 동역학적 분열과 융합 및 미토콘드리아의 분해와 관련된 새로운 요소를 찾아내는 것은 정상적인 미토콘드리아의 질을 유지하는 매커니즘을 밝히는데 중요하다.

이에 본 연구자는 미토콘드리아의 분열과 분해에 중요한 역할을 하는 FKBP8(FK506-binding-protein 8)을 새롭게 찾아내어 보고한다. FKBP8 단백질은 세포 내에서 과발현되면 미토콘드리아의 분해를 일으키고 자가포식과 관련된 단백질인 GFP-LC3 및 GFP-GABARAPL1, 2를 미토콘드리아로 유도하였다. FKBP8의 Deletion mapping을 통해 LC3가 미토콘드리아로 유도되는 데에는 FKBP8의 N-terminal이 반드시 필요함을 확인하였다. FKBP8의 N-terminal에는 LC3와

결합하는데 중요하다고 알려진 LIR (LC3-Interacting-Region)이 LIR#1 ($_{24}$FEVL$_{27}$) 과 LIR#2 ($_{93}$WLDI$_{96}$)의 두 곳에 존재하며 두 LIR 중에서 LIR#1이 LC3와 결합하는데 필수적이었다. 또한, FKBP8 단백질을 세포 내에서 과발현시키면 미토콘드리아의 단편화 역시 유도하는 것을 확인할 수 있었는데, FKBP family의 다른 10가지의 단백질의 과발현으로는 미토콘드리아의 단편화를 유도하지 못하는 것을 관찰함으로써 FKBP family 중에서도 FKBP8의 고유의 특징임을 확인하였다. 반대로 FKBP8을 RNA interference를 통해 발현을 감소시키면 미토콘드리아의 숫자와 부피가 증가함을 관찰하였다. 전자현미경으로 직접적으로 FKBP8의 발현이 감소한 세포의 미토콘드리아를 관찰한 결과 이 세포에서 미토콘드리아가 거대해져 있으며 더 많은 수의 미토콘드리아가 있었다. FKBP8으로 인한 미토콘드리아의 단편화와 분해는 기존에 관련되었다고 보고된 Drp1, BNIP3, NIX와는 관련이 없이 발생하며, 특이하게도 FKBP8의 LC3와 결합하지 않는 LIR#2를 mutagenesis 시켰을 때, 미토콘드리아의 단편화가 감소함을 확인하였다. 미토콘드리아의 단편화와 관련하여 기존에 알려진 미토콘드리아의 분열 요소들 가운데 FIS1이 FKBP8과 결합함을 확인하고, FKBP8의 LIR#2의 point mutation이나 TPR domain을 없앤 경우 이 FIS1과의 결합이 저해되었다. 또한, 저산소조건에서

FKBP8의 발현양의 감소가 미토콘드리아의 분열과 분해를 저해함을 확인할 수 있었다.

정리하자면, 본 연구는 미토콘드리아의 분열과 분해에 관여하는 FKBP8을 새롭게 찾아내었고, FKBP8의 LIR#1은 미토콘드리아의 분해를 매개하며 LIR#2나 TPR domain은 미토콘드리아의 분열 요소인 FIS1과의 결합에 관여함을 밝혔다.