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Relationship between OPA1 and cardiolipin in mitochondrial inner-membrane fusion☆

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ABSTRACT

Mitochondria are highly dynamic organelles that undergo frequent fusion and fission. The large GTPase optic atrophy 1 (OPA1) is identified as a core component of inner membrane (IM) fusion. OPA1 exists as the membrane-anchored L-OPA1 and the proteolytically cleavage soluble S-OPA1. Recently, we showed that OPA1 and mitochondria-localized lipid cardiolipin (CL) cooperate in heterotypic IM fusion [Ban et al., Nat. Cell Biol. 19 (2017) 856–863]. We reconstituted an *in vitro* membrane fusion reaction using purified human L-OPA1 and S-OPA1 expressed in silkworm and found that L-OPA1 on one side of the membrane and CL on the other side were sufficient for mitochondrial fusion. L-OPA1 is the major fusion-prone factor in heterotypic fusion. However, the role of S-OPA1 remains unknown as S-OPA1 promoted L-OPA1-dependent heterotypic membrane fusion and homotypic CL-containing membrane fusion, but S-OPA1 alone was not sufficient for heterotypic membrane fusion. L-OPA1- and CL-mediated heterotypic mitochondrial fusion was confirmed in living cells, but tafazzin (Taz1), the causal gene product of Barth syndrome, was not essential for mitochondrial fusion. Taz1-dependent CL maturation might have other roles in the remodeling of mitochondrial DNA nucleoids.

1. Introduction

Mitochondria play an essential role in ATP production by oxidative phosphorylation, phospholipid metabolism, and apoptosis. Early studies using electron microscopy demonstrated mitochondrial ultrastructural hallmarks such as the double-membrane structure composed of an outer membrane (OM) and inner membrane (IM) and folded narrow tubular structures (*i.e.*, cristae). Recent studies using live imaging of cultured cells have demonstrated that mitochondria are highly dynamic organelles that undergo frequent fusion and fission [1,2].

Mitochondrial morphology is regulated by the balance between fusion and fission [3] (Fig. 1A). When fusion is suppressed, small and fragmented mitochondria are observed. When fission is suppressed, elongated, interconnected mitochondrial networks are observed. A balance between fusion and fission is required for the control of mitochondrial size, position, and shape in the cell to maintain mitochondrial quality and cellular function. Recent findings show that impairment of fusion or fission is observed within some neurodegenerative and metabolic diseases [4,5].

Three types of high molecular weight GTPases have been identified as fusion and fission regulators [6,7] (Fig. 1B). These GTPases are evolutionarily well conserved from yeast to mammals. Mitochondrial fusion is a multi-step process consisting of OM fusion followed by IM fusion [8,9]. In mammals, OM fusion is mediated by OM-localized GTPases, mitofusins (Mfn1 and Mfn2), and IM fusion is mediated by inter-membrane space localized GTPases, optic atrophy 1 (OPA1). OPA1 exists as both a long and a short isoform. Following mitochondrial transport, proteolytic processing produces the IM anchored long-form OPA1 (L-OPA) and the soluble short-form OPA1 (S-OPA1).

These GTPases have been identified as causal gene products of

Abbreviations: OPA1, optic atrophy 1; IM, inner membrane; OM, outer membrane; CL, cardiolipin; KO, knockout; Mfns, mitofusins; MEF, mouse embryonic fibroblast; mtDNA, mitochondrial DNA; Drp1, dynamin-related protein 1; Mff, mitochondrial fission factor; MiD49/MIEF2, mitochondrial dynamics protein of 49 kDa/mitochondrial elongation factor 2; MID51/MIEF1, mitochondrial dynamics protein of 51 kDa/mitochondrial elongation factor 1; ER, endoplasmic reticulum; HR, heptad repeat; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; BmNPV, *Bombyx mori* nucleopolyhedrovirus; DDM, dodecyl maltoside; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-PE; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; FRET, fluorescence resonance energy transfer; CLS1, CL synthase; Taz1, tafazzin; RNAi, RNA-mediated interference; siRNAs, small interfering RNAs; KD, knockdown; HVJ, hemagglutinating virus of Japan

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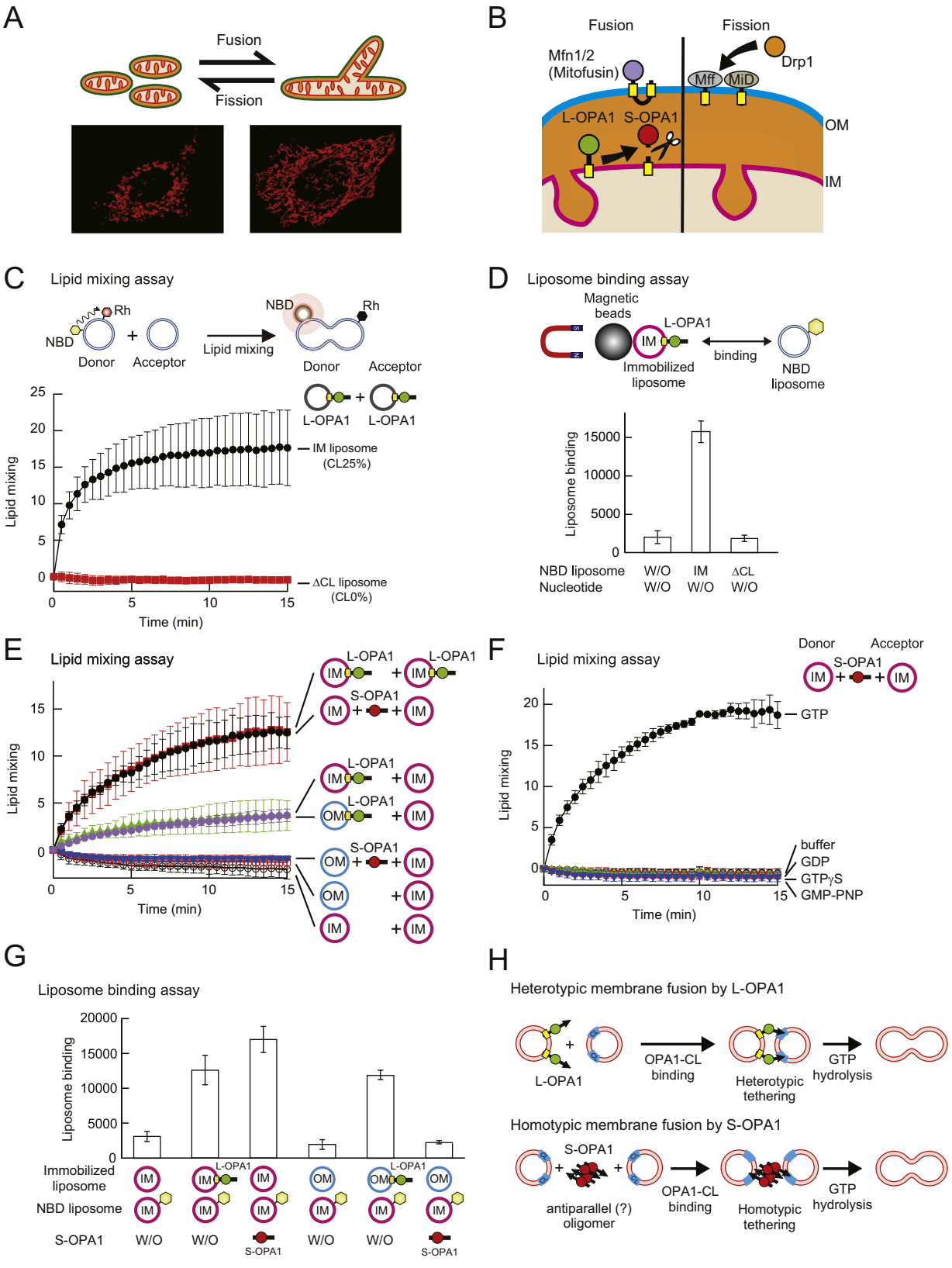

Fig. 1. OPA1-mediated membrane fusion *in vitro*. (A) Mitochondrial morphology is regulated by the balance between fusion and fission. (B) Membrane topology of fusion and fission regulators in mammalian cells. (C) Homotypic L-OPA1-mediated membrane fusion. The requirement for CL was analyzed in the homotypic condition. (D) Liposomes binding between IM liposomes containing L-OPA1 and NBD-labeled liposomes in the absence of nucleotides. (E) S-OPA1-mediated membrane fusion. Membrane fusion between various liposomes in the presence of S-OPA1. (F) Effects of various guanine nucleotides on homotypic S-OPA1-mediated membrane fusion. (G) Liposome binding between OM or IM liposomes and NBD-labeled IM liposomes in the presence of S-OPA1. The effect of CL was analyzed in the absence of nucleotides. (H) Models for membrane fusion by L-OPA1 and S-OPA1. Top: L-OPA1-CL binding promotes the tethering of opposite membranes, and GTP hydrolysis mediates membrane fusion. Bottom: a higher intermolecular complex of S-OPA1 leads to homotypic membrane tethering and fusion. All data were the average of three independent measurements, and error bars represent standard deviations.

neurodegenerative disorders. Mutations in Mfn2 lead to Charcot–Marie–Tooth neuropathy type 2a [10], and mutations in OPA1 lead to dominant optic atrophy type I [11], suggesting that mitochondrial fusion is important in neural function. Gene knockout (KO) for Mfns [1] and OPA1 [12] is lethal in embryos. Skeletal muscle-specific KO in mice for Mfns [13], OPA1 [14], neuron-specific Mfns-KO [15], and cardiac muscle-specific Mfns-KO [16] result in severe pathogenesis due to mitochondrial dysfunction. These studies have provided clear evidence that mitochondrial fusion is essential not only in the maintenance of mitochondrial morphology but also in mitochondrial function. In addition, mouse embryonic fibroblast (MEF) cells lacking Mfns or OPA1 lost mitochondrial DNA (mtDNA), which encodes several important subunits of the electron transport chain complexes, suggesting that mitochondrial fusion plays an important role in the stabilization of mtDNA [13].

Mitochondrial fission is regulated by cytosolic GTPase, dynamin-related protein 1 (Drp1). During mitochondrial fission, the OM-localized protein, mitochondrial fission factor (Mff) [17,18], mitochondrial dynamics protein 49 kDa/mitochondrial elongation factor 2 (MiD49/MIEF2) [19], and mitochondrial dynamics protein 51 kDa/mitochondrial elongation factor 1 (MiD51/MIEF1) [19,20] control Drp1 recruitment to the fission site to facilitate fission. It has been suggested that before Drp1 recruitment to the OM membrane, tubules of the endoplasmic reticulum (ER) wrap around mitochondria and mark the mitochondrial fission site [21,22]. Some dominant negative mutations within Drp1 cause neonatal death with abnormal mitochondrial fission and brain development [23,24]. In mice studies, gene KO for Drp1 leads to the death of embryos [25]. Neuron-specific Drp1-KO [25] and cardiac muscle-specific Drp1-KO [26] are lethal after birth owing to severe neurodegeneration and impairment of cardiac muscle contraction, respectively.

Some *in vitro* studies provide insight into how GTPases mediate mitochondrial fusion and fission. Structural analysis indicated that the cytosolic C-terminal hydrophobic heptad repeat (HR) of Mfn1 forms homotypic dimers with an antiparallel coiled-coil structure and results in the tethering of adjacent but opposite membranes [27]. A trans HR assembly motif is also essential for membrane fusion mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [28]. It has recently been proposed that Mfn1 in opposite membranes forms homotypic dimers through interactions with the GTPase domain and nucleotide binding to follow tethering [29,30]. This process is related to the ER fusion mediated by the ER-bound GTPase, atlastin [31]. It is clear that mitofusin brings opposite OMs closer, but its function in complete fusion remains unknown.

Recombinant Drp1 assembles into a ring on the surface of the liposome and mediates membrane tubulation [32]. By its overall structural similarity to dynamin 1 [33,34], Drp1 assembly on the membrane surface stimulates GTPase activity, leading to the conformational change that might trigger membrane constriction and fission. The effects of interactions between Drp1 and Mff or MiD proteins during the fission reaction remain important issues to be addressed in the future. Owing to the difficulty of preparing active recombinant fusogenic GTPases, it remains unclear how fusogenic GTPases mediate mitochondrial OM and IM fusion. Recently, we have developed methods of expressing and purifying human OPA1 using the *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid-silkworm expression system and reconstituted a membrane fusion assay [35]. Using this approach, we focus on the effects of mitochondria-localized lipid cardiolipin (CL) on L-OPA1- and S-OPA1-mediated homotypic membrane fusion.

2. Materials and methods

2.1. Recombinant L-OPA1 and S-OPA1 expression and purification

Both L-OPA1 and S-OPA1 were expressed in silkworm fat bodies using the BmNPV bacmid-silkworm expression system [36]. L-OPA1 and S-OPA1 were purified from isolated silkworm fat bodies, as previously described [35,37]. The recombinant BmNPV bacmid DNA containing L-OPA1 or S-OPA1 was injected directly into the dorsal side of a silkworm larva with the lipofection reagent DMRIE-C (Life Technologies). After 7 days of culture, infected fat bodies were isolated manually and homogenized in ice-cold PB buffer (50 mM sodium phosphate, pH 8, 500 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, 0.5% sodium thiosulfate). Following sonication, disrupted fat bodies were centrifuged at 14,000*g* for 1 h. The pellet fraction was lysed with PB buffer containing 1% dodecyl maltoside (DDM) (Dojindo) for 2 h at 4 °C with gentle agitation and centrifuged at 14,000*g* for 30 min. After the addition of Ni Sepharose 6 Fast Flow beads (GE Healthcare), the supernatant was incubated overnight at 4 °C. After extensive washing with RB buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10% glycerol, 1 mM DTT) containing 0.1% DDM and 20 mM imidazole, proteins were eluted from Ni Sepharose 6 beads with RB buffer containing 0.1% DDM and 250 mM imidazole. For S-OPA1, DDM and imidazole were removed by dialysis with Bio-beads SM-2 (Bio-rad).

2.2. Preparation of liposomes containing L-OPA1

Liposomes containing L-OPA1 were prepared by the detergent-dialysis method, as previously described [35]. For the detergent exchange, purified L-OPA1 fractions were diluted 10-fold with RB buffer with 0.1% DDM and applied into the Ni Sepharose 6 column. After washing with 20 column volumes of RB containing 2.5% MEGA-8 (Dojindo), L-OPA1 was eluted with RB containing 2.5% MEGA-8 and 250 mM imidazole.

Nonfluorescent lipids were purchased from Avanti Polar Lipids. Fluorescent lipids *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Molecular Probes. The lipid compositions of liposomes were designed to mimic the lipid composition of the mitochondrial IM and OM [38]. The lipid compositions of the liposomes were, by percent-molar, as follows: IM liposomes (CL $(18:1)_4$:POPC:POPE:soy-PI; 25:42:25:8), ΔCL liposomes (POPC:POPE:soy-PI; 67:25:8), OM liposomes (CL$(18:1)_4$:POPC:POPE:soy-PI; 4:60:27:9). For donor liposomes, 3% of POPE in IM, ΔCL, or OM liposomes was substituted for 1.5% NBD-PE and 1.5% Rh-PE. Lipids were mixed with the indicated ratio in chloroform and dried with N_2 gas. After further drying in a vacuum, the dried lipid films were dissolved in RB containing 5% MEGA-8 to a total lipid concentration of 8 mM. L-OPA1 was added to a concentration of 2 μM, and the detergent–lipid–protein mixture containing 3.2% MEGA-8 and 2 mM lipids was incubated for 2 h at 4 °C. Liposomes containing L-OPA1 were obtained by removal of MEGA-8 by dialysis against RB at 4 °C.

2.3. Membrane fusion assay

Membrane fusion was analyzed on the basis of the well-known lipid mixing reaction as described previously [28,39] with the following modifications [35]. The fluorescence-labeled donor liposomes containing L-OPA1 (L-OPA1 and liposomes at concentrations of 200 nM and 200 μM, respectively) and the nonfluorescence-labeled acceptor liposomes containing L-OPA1 (L-OPA1 and liposomes at concentrations of 500 nM and 500 μM, respectively) were mixed in RB buffer in a black 384-well plate and preincubated for 10 min at 30 °C in the infinite F-200 plate reader (TECAN). GTP/$MgCl_2$ was then added to the mixture at a concentration of 5 mM to initiate reactions at 30 °C. NBD fluorescence was measured at 540 nm with excitation at 465 nm to monitor lipid mixing. To evaluate fusion efficiency, the absolute NBD fluorescence was determined by adding Triton X-100 at a concentration of 0.4%. Data were normalized to the percentage of total NBD fluorescence as described previously [28,39]. For S-OPA1-mediated membrane fusion, the fluorescence-labeled donor liposomes (200 μM), the nonfluorescence-labeled acceptor liposomes (500 μM) and S-OPA1

(700 nM) were mixed in RB buffer and fusion reaction was measured in the same manner as for L-OPA1.

2.4. Liposome binding assay

Liposome binding was analyzed using methods described previously [40] with the following modifications [35]. For the immobilization of OM or IM liposomes containing L-OPA1 (L-OPA1 and liposomes at concentrations of 150 nM and 150 μM, respectively) to the surface of avidin-coated M-280 Dynabeads (Thermo Fisher Scientific), 3% of POPE in OM or IM liposomes was substituted for 3% biotinyl-PE. After adding NBD-labeled liposomes (150 μM) in the absence of nucleotides, the sample mixture was gently agitated for 30 min at room temperature. Dynabeads were isolated with a magnet and washed with RB three times, followed by resuspension in RB containing 0.4% Triton X-100. The interaction between L-OPA1 and the membrane was quantified by measuring NBD fluorescence at 540 nm with excitation at 465 nm from bound NBD-labeled liposomes with a plate reader. For S-OPA1-mediated liposome binding, S-OPA1 (150 nM) and NBD-labeled liposomes (150 μM) were mixed with the immobilized OM or IM liposomes (150 μM) on the surface of Dynabeads and the interaction was quantified in the same manner as for L-OPA1.

2.5. Cell culture, RNAi, imaging, and quantitative PCR

HeLa cells expressing green fluorescent protein (GFP) or DsRed (red fluorescent protein; RFP) fused with a mitochondrial-targeting signal (mit-GFP or mit-RFP, respectively) [41] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma). For RNA-mediated interference (RNAi), small interfering RNAs (siRNAs) were introduced into HeLa cells by reverse transfection with Lipofectamine RNAiMax (Invitrogen). siRNAs designed for human OPA1, Drp1, CLS1, and Taz1 have been described previously [35,42,43]. For the analysis of mitochondrial morphology and nucleoid size, HeLa cells expressing mit-RFP were stained with 100,000-fold diluted SYBR Green I (Invitrogen) [42] and observed under a fluorescent microscope (IX81; Olympus). Colocalization of mitochondrial nucleoid-associated SYBR Green I fluorescence and mitochondrial mit-RFP-associated fluorescence was evaluated with the MetaMorph software (Molecular Devices). For the analysis of mtDNA content, DNA was extracted from HeLa cells and quantitative PCR as previously described [42] with a StepOne Plus (Thermo).

2.6. Mitochondrial fusion assay in cultured cells

Mitochondrial fusion assay in HeLa cells was performed, as previously described [35,41]. HeLa cells expressing mit-GFP or mit-RFP were treated with siRNA twice for 6 days, and cells were then co-cultured in a glass-bottomed dish. Cells were fused by hemagglutinating virus of Japan (HVJ) envelope (GenomeONE-CF, Ishihara Sangyo). After 6 h of incubation, mitochondrial fusion was analyzed as the co-localization of GFP-labeled mitochondria (mit-GFP) and RFP-labeled mitochondria (mit-RFP).

3. Results and discussion

3.1. L-OPA1 and CL mediate membrane fusion

To better understand OPA1 function in fusion, we developed methods to obtain large amounts of recombinant L-OPA1 [35,37]. Purified L-OPA1 and mitochondrial IM contact sites mimicking liposomes, which contain 25% CL, were mixed in detergent, and liposomes containing L-OPA1 were prepared by dialysis in detergent-free buffer. We succeeded in reconstituting the membrane fusion reaction (reported in [35]) using a well-established fluorescence resonance energy transfer (FRET)-based lipid mixing assay [28,39]. After the addition of GTP, efficient membrane fusion was observed in liposomes containing L-OPA1 depending on GTP hydrolysis whereas membrane fusion was not observed in protein-free liposomes, suggesting that L-OPA1 alone can cause membrane fusion *in vitro* (Fig. 1C, E) [35].

CL is the most abundant negatively charged lipid in the IM (18–25% of IM lipids), although only trace amounts of CL are located in the mitochondrial OM (4% of total OM lipids) [38]. CL has a unique chemical structure with two phosphate head groups and four acyl chains that generate various functions, including stabilization of the respiratory complex [44], regulation of cytochrome *c* release [45], mitochondrial dynamics [46], and mitophagy as a receptor of LC3 [47]. When CL was removed from IM-OPA1, no membrane fusion was observed even in the presence of L-OPA1 on both sides (Fig. 1C). We previously showed that membrane fusion was not observed in OM-mimicked liposomes containing trace amounts of CL, even in the presence of L-OPA1 [35]. These results suggest that CL is a critical component of L-OPA1-mediated membrane fusion.

We previously demonstrated a unique feature of the L-OPA1-mediated membrane fusion reaction from a series of *in vitro* fusion analyses. When OM or IM liposomes containing L-OPA1 were mixed with protein-free IM liposomes, partial but significant fusion was observed (Fig. 1E), as reported previously [35]. In contrast, no fusion was observed under CL reduction in protein-free liposomes. These results indicate that L-OPA1 on one side of the membrane and CL on the other side are sufficient for fusion (Fig. 1H), as reported previously [35]. To confirm CL function in L-OPA1-mediated membrane fusion, we performed a liposome binding assay. We found that liposome binding was dependent on lipid composition. Binding was observed only for NBD-labeled liposomes with CL (Fig. 1D). We previously found that OM liposomes containing L-OPA1 bound to NBD-labeled IM liposomes (Fig. 1G) [35], indicating that CL on the same side of L-OPA1 is not necessary for liposome binding. When CL was replaced with other negatively charged phospholipids such as phosphatidic acid, phosphatidylserine, or phosphatidylglycerol, reduced liposome binding was detected [35].

Taken together, these studies suggest that CL acts as a specific binding site for OPA1 during membrane tethering prior to membrane fusion. The domain next to the GTPase domain of L-OPA1 and the proper CL composition of the acyl chain would allow specific interactions between L-OPA1 and CL [35]. Therefore, L-OPA1 has two membrane-binding domains, a transmembrane domain at the N-terminus and a CL-binding domain at the C-terminus (Fig. 1H).

3.2. Role of S-OPA1 in membrane fusion

In the steady state, L-OPA1 coexists with S-OPA1 lacking the N-terminal transmembrane domain (Fig. 1B). When cells were treated with protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), the ensuing loss of membrane potential caused mitochondrial fragmentation with complete proteolytic conversion of L-OPA1 to S-OPA1, suggesting that OPA1 conversion is related to the regulation of mitochondrial fusion and mitochondrial quality control [48]. However, the role of S-OPA1 in mitochondrial fusion remains controversial. A combination of L- and S-OPA1 expression was reported to be required for optimal fusion in OPA1-null MEF cells [49].

To understand the function of S-OPA1 in membrane fusion, we prepared a recombinant S-OPA1 using the silkworm expression system in the same manner as for L-OPA1 [35]. We previously showed that addition of detergent-free S-OPA1 enhanced L-OPA1-mediated heterotypic fusion [35]. However, S-OPA1 alone failed to mediate heterotypic membrane fusion between CL-containing and CL-reduced liposomes (Fig. 1E), as reported previously [35]. We further examined the fusogenic property of S-OPA1 in the homotypic CL condition. When S-OPA1 was added to the homotypic fusion reaction between IM liposomes, membrane fusion was observed in a GTP hydrolysis-dependent manner (Fig. 1E, F).

To better understand CL function, we performed a liposome binding assay. IM liposomes bound to immobilized IM liposomes in the presence of S-OPA1, whereas IM liposomes did not bind to immobilized OM liposomes even in the presence of S-OPA1 (Fig. 1G), suggesting that S-OPA1 bridges the opposite membrane in a CL-dependent manner. This CL-dependent membrane binding is consistent with a previous study, but membrane fusion was not consistent in the study [50]. It is not clear whether this discrepancy results from the different fusion assay used or whether the increased NBD fluorescence in this study reflects the lipid transfer reaction.

One of our speculation is that S-OPA1 may form a higher intermolecular complex that may have several CL-binding domains. Higher CL-binding capacity may mediate homotypic membrane tethering and fusion (Fig. 1H). However, we do not believe that these S-OPA1 properties reflect the physiological properties of S-OPA1, because S-OPA1 is only produced from L-OPA1 after anchoring to the IM in the correct direction (*i.e.*, topology). Considering that expression of OPA1-spliced forms containing exon 4b in OPA1-null MEF cells yields only S-OPA1 and results in loss of fusion activity [49,51,52], we need to carefully consider whether recombinant soluble S-OPA1 mimics the physiological condition. Therefore, additional analysis is needed to understand the molecular properties of S-OPA1 (*e.g.*, assembly state and orientation), both in solution and on the membrane.

3.3. Role of CL for mitochondrial fusion in living cells

Our liposome-based *in vitro* assay showed that CL is required for L-OPA1-mediated fusion. To examine the function of CL for mitochondrial fusion in living cells, HVJ-induced cell fusion [35,41] was used to investigate mitochondrial fusion. In mammals, CL synthesis occurs in the mitochondrial IM [53,54]. *De novo* CL is synthesized from cytidine diphosphate diacylglycerol and phosphatidylglycerol by CL synthase (CLS1). The maturation of CL is caused by phospholipid transferase tafazzin (Taz1)-catalyzed acyl chain remodeling. OPA1, CLS1, and Taz1 were repressed by RNAi-mediated knockdown (KD) in HeLa cells. We previously demonstrated a unique feature of mitochondrial IM fusion using cultured cell hybrids. When OPA1-KD cells and control HeLa cells were fused, mitochondrial fusion was observed (Fig. 2A), as reported previously [35]. Significant fusion was also observed in cell hybrids with CLS1-KD and OPA1-KD cells [35]. However, when HeLa cells in which both CLS1 and OPA1 were suppressed fused with control cells, fusion was not observed [35]. These results show that for the complete isolation of damaged mitochondria from the active mitochondrial network, inactivation of OPA1 is insufficient. We suggest that CL might act as another determining factor in selective mitochondrial fusion prior to mitophagy, although further study is required to analyze the distribution of CL in damaged mitochondria. In contrast, Taz1-KD did not inhibit mitochondrial fusion (Fig. 2B), suggesting that Taz1-catalyzed CL maturation is dispensable for selective mitochondrial fusion.

3.4. Role of CL maturation in mitochondrial morphology and mtDNA distribution

We examined the contribution of Taz1-catalyzed CL maturation in mitochondrial morphology and mtDNA distribution. In mammals, multicopy mtDNA are assembled with mtDNA-associated proteins such as mitochondrial transcription factor A and Twinkle to form nucleoid structures [55]. In control cells, the SYBR Green I staining showed that nucleoids were widely dispersed in mitochondria (Fig. 3A). Most of the SYBR Green I fluorescence signals (> 95.9%) were colocalized with mitochondrial mit-RFP-associated fluorescence signals, indicating that nucleoids were specifically stained. The extent of colocalization is indicated for each condition in Fig. 3A. In Drp1-KD cells, large nucleoid clusters within the elongated mitochondrial network were observed (Fig. 3A, B) [42]. Partial nucleoid clustering can also be observed by overexpressing Mfn1 [42], suggesting that balanced fusion and fission

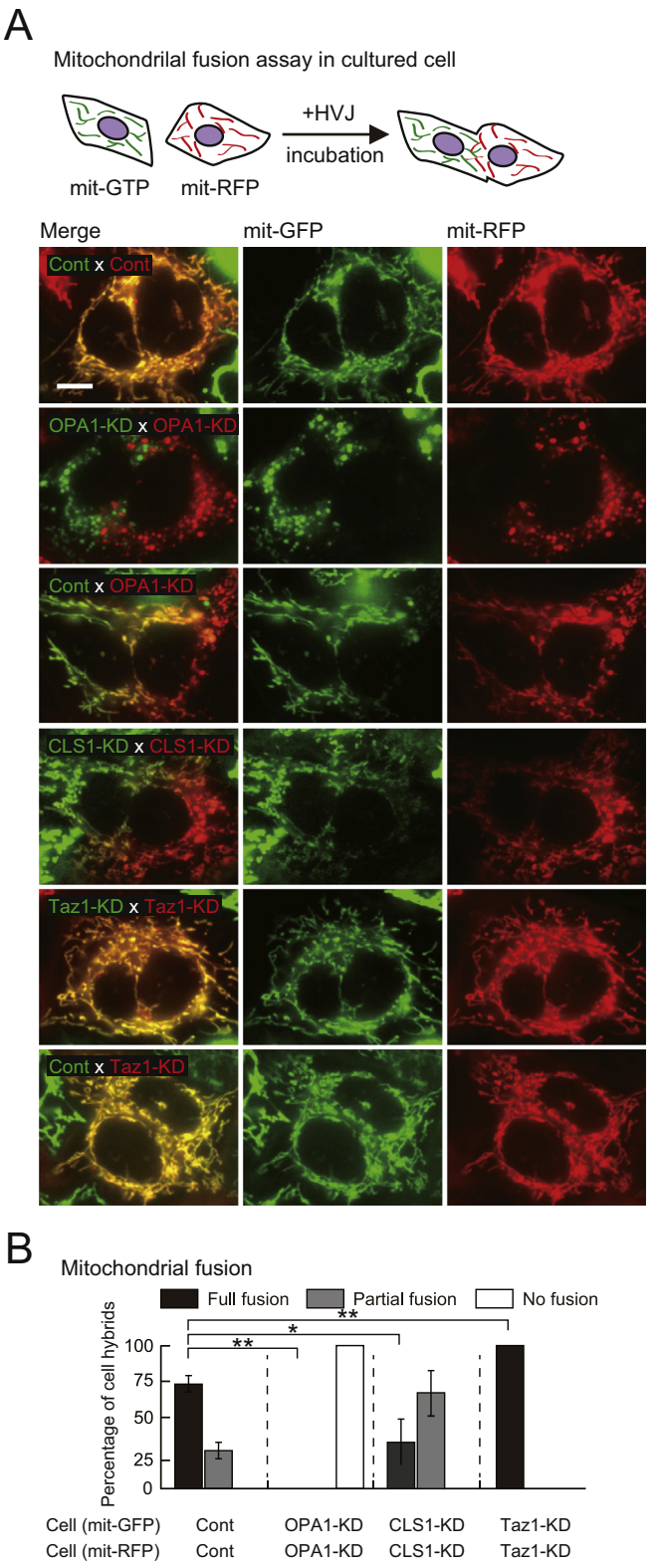

Fig. 2. Taz1 is not required for mitochondrial fusion. (A) Representative images of mitochondrial fusion in HeLa cells treated with siRNAs for OPA1, CLS1, or Taz1, as indicated in combinations. Mitochondrial fusion was analyzed in cell hybrids from cells expressing mit-GFP and mit-RFP. Cells were fused using HVJ envelope and fusion efficiency was analyzed by fluorescence microscopy. (B) HeLa cell hybrids were classified as cell hybrids with full fusion, partial fusion, or no fusion from three independent counts (each ~10 cell hybrids). $^{*}P < 0.05$, $^{**}P < 0.01$, paired *t*-test. Scale bar: 10 μm. Error bars represent standard deviations.

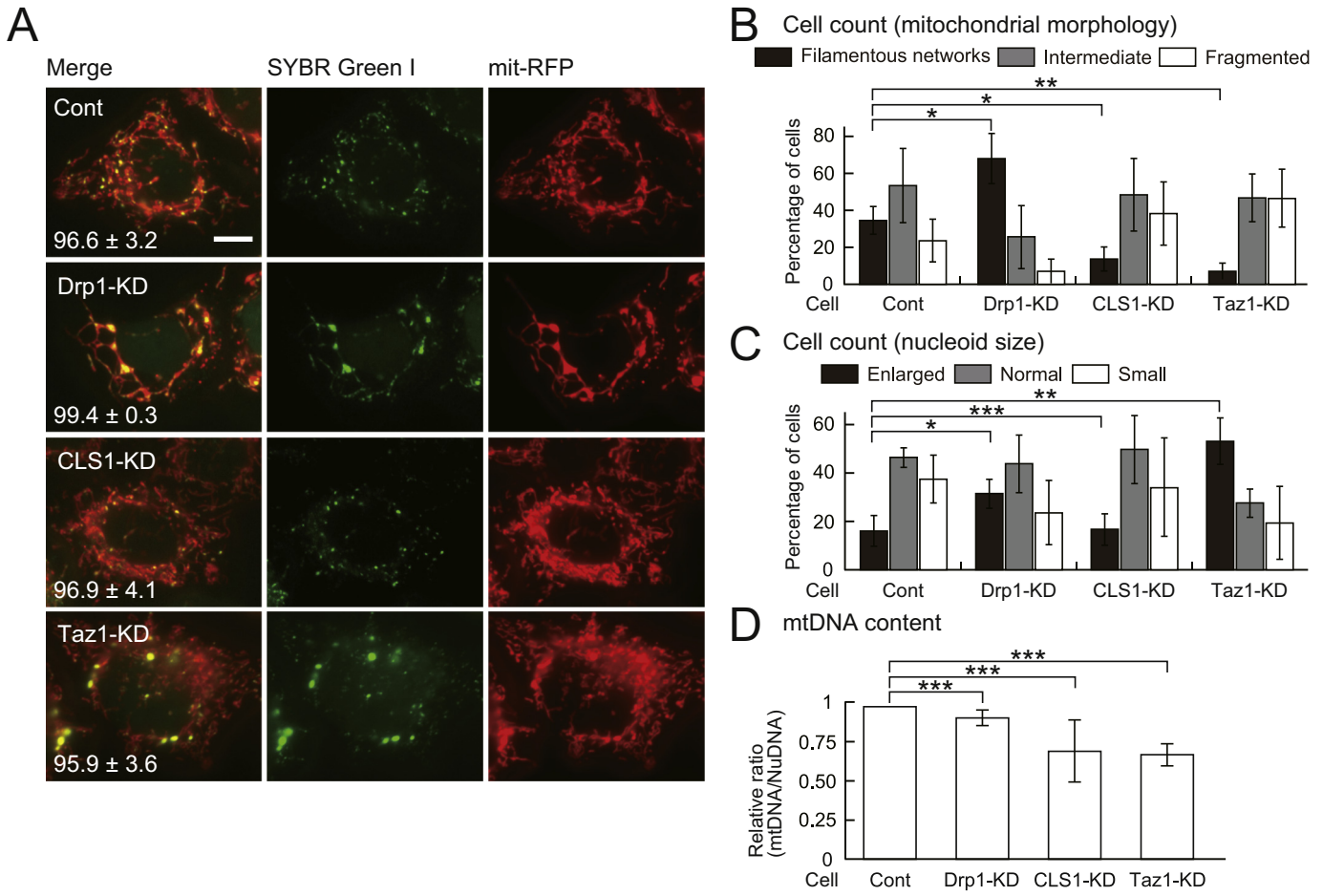

Fig. 3. Enlarged nucleoids in Taz1-deficient cells. (A) Representative images of mitochondrial morphology and nucleoids in HeLa cells treated with indicated siRNA. HeLa cells expressing mit-RFP were stained with SYBR Green I and analyzed by fluorescence microscopy. The numbers indicate the extent of colocalization of SYBR Green I with the mit-RFP signal (mean ± standard deviation, n = 3). Scale bar: 10 μm. (B) HeLa cells treated as (A) were classified as cells with filamentous networks, intermediate or fragmented mitochondrial structures from three independent cell counts (each ~100 cells). (C) HeLa cells treated as (A) were classified as cells with enlarged, normal, or small nucleoids from three independent cell counts (each ~100 cells). (D) The mtDNA content of each cell was analyzed by quantitative PCR. The relative ratio indicates the amount of mtDNA per nuclear DNA. Three independent experiments were performed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P > 0.05$, paired t-test. Error bars represent standard deviations.

may be related to the formation of nucleoid clusters. Here we found that large nucleoid clusters were observed in Taz1-KD cells (Fig. 3A, C), although Taz1-KD did not inhibit mitochondrial fusion (Fig. 2B) and did not induce mitochondrial elongation (Fig. 3B). In Taz1-KD cells, the total mtDNA content did not increase but instead decreased (Fig. 3D).

These results suggest that Taz1-catalyzed CL maturation is involved in proper mtDNA distribution, but its regulation machinery might be different from GTPase, including OPA1- and Drp1-mediated mitochondrial fusion and fission. In CLS1-KD cells, mitochondrial fusion was repressed, but mitochondrial morphology was only partly affected (Figs. 2B and 3A, B). It has recently been reported that inhibiting the CL synthesis pathway in the nematode *Caenorhabditis elegans* and cultured mammalian cells led to mitochondrial elongation [56], supporting our conclusion that CL has several functions in the maintenance and regulation of mitochondrial activity and morphology.

3.5. Conclusions

We demonstrated the relationship between OPA1 and CL in mitochondrial IM fusion. To evaluate OPA1-mediated mitochondrial IM fusion, we developed methods of expressing and purifying active human OPA1 using the BmNPV bacmid-silkworm expression system. Because silkworm expression is well suited for the analysis of mitochondrial membrane proteins, the present method can be applied to the analysis of Mfns-mediated membrane fusion. Additional studies are required to completely understand mitochondrial fusion. We showed that GTP hydrolysis is needed for OPA1-mediated fusion. However, it is important to fully address how GTP hydrolysis leads to complete fusion. We speculate that CL acts as a determining factor for the isolation of damaged mitochondria. It is therefore important to analyze the spatiotemporal arrangement of CL in damaged mitochondria. We suggest that L-OPA1 may have a CL-binding domain at the C-terminus. Therefore, the CL-binding domain may be applicable for developing novel CL markers in living cells.

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References

[1] H. Chen, S.A. Detmer, A.J. Ewald, E.E. Griffin, S.E. Fraser, D.C. Chan, Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development, J. Cell Biol. 160 (2003) 189–200.

[2] N. Ishihara, A. Jofuku, Y. Eura, K. Mihara, Regulation of mitochondrial morphology by membrane potential, and DRP1-dependent division and FZO1-dependent fusion reaction in mammalian cells, Biochem. Biophys. Res. Commun. 301 (2003) 891–898.

[3] H. Sesaki, R.E. Jensen, Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape, J. Cell Biol. 147 (1999) 699–706.

[4] J. Nunnari, A. Suomalainen, Mitochondria: in sickness and in health, Cell 148 (2012) 1145–1159.

[5] L. Pernas, L. Scorrano, Mito-morphosis: mitochondrial fusion, fission, and cristae remodeling as key mediators of cellular function, Annu. Rev. Physiol. 78 (2016) 505–531.

[6] N. Ishihara, H. Otera, T. Oka, K. Mihara, Regulation and physiologic functions of GTPases in mitochondrial fusion and fission in mammals, Antioxid. Redox Signal. 19 (2013) 389–399.

[7] J.A. McNew, H. Sondermann, T. Lee, M. Stern, F. Brandizzi, GTP-dependent membrane fusion, Annu. Rev. Cell Dev. Biol. 29 (2013) 529–550.

[8] S. Meeusen, J.M. McCaffery, J. Nunnari, Mitochondrial fusion intermediates revealed *in vitro*, Science 305 (2004) 1747–1752.

[9] S. Hoppins, F. Edlich, M.M. Cleland, S. Banerjee, J.M. McCaffery, R.J. Youle, J. Nunnari, The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes, Mol. Cell 41 (2011) 150–160.

[10] S. Züchner, I.V. Mersiyanova, M. Muglia, N. Bissar-Tadmouri, J. Rochelle, E.L. Dadali, M. Zappia, E. Nelis, A. Patitucci, J. Senderek, Y. Parman, O. Evgrafov, P.D. Jonghe, Y. Takahashi, S. Tsuji, M.A. Pericak-Vance, A. Quattrone, E. Battaloglu, A.V. Polyakov, V. Timmerman, J.M. Schroder, J.M. Vance, Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A, Nat. Genet. 36 (2004) 449–451.

[11] C. Delettre, G. Lenaers, J.M. Griffoin, N. Gigarel, C. Lorenzo, P. Belenguer, L. Pelloquin, J. Grosgeorge, C. Turc-Carel, E. Perret, C. Astarie-Dequeker, L. Lasquellec, B. Arnaud, B. Ducommun, J. Kaplan, C.P. Hamel, Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy, Nat. Genet. 26 (2000) 207–210.

[12] V.J. Davies, A.J. Hollins, M.J. Piechota, W. Yip, J.R. Davies, K.E. White, P.P. Nicols, M.E. Boulton, M. Votruba, Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function, Hum. Mol. Genet. 16 (2007) 1307–1318.

[13] H. Chen, M. Vermulst, Y.E. Wang, A. Chomyn, T.A. Prolla, J.M. McCaffery, D.C. Chan, Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations, Cell 141 (2010) 280–289.

[14] C. Tezze, V. Romanello, M.A. Desbats, G.P. Fadini, M. Albiero, G. Favaro, S. Ciciliot, M.E. Soriano, V. Morbidoni, C. Cerqua, S. Loefler, H. Kern, C. Franceschi, S. Salvioli, M. Conte, B. Blaauw, S. Zampieri, L. Salviati, L. Scorrano, M. Sandri, Age-associated loss of OPA1 in muscle impacts muscle mass, metabolic homeostasis, systemic inflammation, and epithelial senescence, Cell Metab. 25 (2017) 1374–1389 (e1376).

[15] H. Chen, J.M. McCaffery, D.C. Chan, Mitochondrial fusion protects against neurodegeneration in the cerebellum, Cell 130 (2007) 548–562.

[16] Y. Chen, Y. Liu, G.W. Dorn 2nd, Mitochondrial fusion is essential for organelle function and cardiac homeostasis, Circ. Res. 109 (2011) 1327–1331.

[17] S. Gandre-Babbe, A.M. van der Bliek, The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells, Mol. Biol. Cell 19 (2008) 2402–2412.

[18] H. Otera, C. Wang, M.M. Cleland, K. Setoguchi, S. Yokota, R.J. Youle, K. Mihara, Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells, J. Cell Biol. 191 (2010) 1141–1158.

[19] C.S. Palmer, L.D. Osellame, D. Laine, O.S. Koutsopoulos, A.E. Frazier, M.T. Ryan, MiD49 and MiD51, new components of the mitochondrial fission machinery, EMBO Rep. 12 (2011) 565–573.

[20] J. Zhao, T. Liu, S. Jin, X. Wang, M. Qu, P. Uhlén, N. Tomilin, O. Shupliakov, U. Lendahl, M. Nistér, Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission, EMBO J. 30 (2011) 2762–2778.

[21] J.R. Friedman, L.L. Lackner, M. West, J.R. DiBenedetto, J. Nunnari, G.K. Voeltz, ER tubules mark sites of mitochondrial division, Science 334 (2011) 358–362.

[22] W.K. Ji, R. Chakrabarti, X. Fan, L. Schoenfeld, S. Strack, H.N. Higgs, Receptor-mediated Drp1 oligomerization on endoplasmic reticulum, J. Cell Biol. 216 (2017) 4123–4139.

[23] H.R. Waterham, J. Koster, C.W. van Roermund, P.A. Mooyer, R.J. Wanders, J.V. Leonard, A lethal defect of mitochondrial and peroxisomal fission, N. Engl. J. Med. 356 (2007) 1736–1741.

[24] J.A. Fahrner, R. Liu, M.S. Perry, J. Klein, D.C. Chan, A novel *de novo* dominant negative mutation in DNM1L impairs mitochondrial fission and presents as childhood epileptic encephalopathy, Am. J. Med. Genet. A 170 (2016) 2002–2011.

[25] N. Ishihara, M. Nomura, A. Jofuku, H. Kato, S.O. Suzuki, K. Masuda, H. Otera, Y. Nakanishi, I. Nonaka, Y. Goto, N. Taguchi, H. Morinaga, M. Maeda, R. Takayanagi, S. Yokota, K. Mihara, Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice, Nat. Cell Biol. 11 (2009) 958–966.

[26] T. Ishihara, R. Ban-Ishihara, M. Maeda, Y. Matsunaga, A. Ichimura, S. Kyogoku, H. Aoki, S. Katada, K. Nakada, M. Nomura, N. Mizushima, K. Mihara, N. Ishihara, Dynamics of mitochondrial DNA nucleoids regulated by mitochondrial fission is essential for maintenance of homogeneously active mitochondria during neonatal heart development, Mol. Cell. Biol. 35 (2015) 211–223.

[27] T. Koshiba, S.A. Detmer, J.T. Kaiser, H. Chen, J.M. McCaffery, D.C. Chan, Structural basis of mitochondrial tethering by mitofusin complexes, Science 305 (2004) 858–862.

[28] T. Weber, B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Sollner, J.E. Rothman, SNAREpins: minimal machinery for membrane fusion, Cell 92 (1998) 759–772.

[29] Y. Qi, L. Yan, C. Yu, X. Guo, X. Zhou, X. Hu, X. Huang, Z. Rao, Z. Lou, J. Hu, Structures of human mitofusin 1 provide insight into mitochondrial tethering, J. Cell Biol. 215 (2016) 621–629.

[30] Y.L. Cao, S. Meng, Y. Chen, J.X. Feng, D.D. Gu, B. Yu, Y.J. Li, J.Y. Yang, S. Liao, D.C. Chan, S. Gao, MFN1 structures reveal nucleotide-triggered dimerization critical for mitochondrial fusion, Nature 542 (2017) 372–376.

[31] X. Bian, R.W. Klemm, T.Y. Liu, M. Zhang, S. Sun, X. Sui, X. Liu, T.A. Rapoport, J. Hu, Structures of the atlastin GTPase provide insight into homotypic fusion of endoplasmic reticulum membranes, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 3976–3981.

[32] C. Fröhlich, S. Grabiger, D. Schwefel, K. Faelber, E. Rosenbaum, J. Mears, O. Rocks, O. Daumke, Structural insights into oligomerization and mitochondrial remodelling of dynamin 1-like protein, EMBO J. 32 (2013) 1280–1292.

[33] M.G. Ford, S. Jenni, J. Nunnari, The crystal structure of dynamin, Nature 477 (2011) 561–566.

[34] K. Faelber, Y. Posor, S. Gao, M. Held, Y. Roske, D. Schulze, V. Haucke, F. Noé, O. Daumke, Crystal structure of nucleotide-free dynamin, Nature 477 (2011) 556–560.

[35] T. Ban, T. Ishihara, H. Kohno, S. Saita, A. Ichimura, K. Maenaka, T. Oka, K. Mihara, N. Ishihara, Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin, Nat. Cell Biol. 19 (2017) 856–863.

[36] M. Kajikawa, K. Sasaki, Y. Wakimoto, M. Toyooka, T. Motohashi, T. Shimojima, S. Takeda, E.Y. Park, K. Maenaka, Efficient silkworm expression of human GPCR (nociceptin receptor) by a *Bombyx mori* bacmid DNA system, Biochem. Biophys. Res. Commun. 385 (2009) 375–379.

[37] T. Ban, N. Ishihara, Expression and purification of recombinant human L-OPA1 using BmNPV bacmid-silkworm expression system, Protoc. Exch. https://doi.org/10.1038/protex.2017.053 2017 (accessed 20 June 2017).

[38] D. Ardail, J.P. Privat, M. Egret-Charlier, C. Levrat, F. Lerme, P. Louisot, Mitochondrial contact sites. Lipid composition and dynamics, J. Biol. Chem. 265 (1990) 18797–18802.

[39] J. Mima, W. Wickner, Complex lipid requirements for SNARE- and SNARE chaperone-dependent membrane fusion, J. Biol. Chem. 284 (2009) 27114–27122.

[40] S. Sugiura, J. Mima, Physiological lipid composition is vital for homotypic ER membrane fusion mediated by the dynamin-related GTPase Sey1p, Sci. Rep. 6 (2016) 20407.

[41] Y. Eura, N. Ishihara, S. Yokota, K. Mihara, Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion, J. Biol. Chem. 134 (2003) 333–344.

[42] R. Ban-Ishihara, T. Ishihara, N. Sasaki, K. Mihara, N. Ishihara, Dynamics of nucleoid structure regulated by mitochondrial fission contributes to cristae reformation and release of cytochrome c, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 11863–11868.

[43] R. Richter-Dennerlein, A. Korwitz, M. Haag, T. Tatsuta, S. Dargazanli, M. Baker, T. Decker, T. Lamkemeyer, E.I. Rugarli, T. Langer, DNAJC19, a mitochondrial cochaperone associated with cardiomyopathy, forms a complex with prohibitins to regulate cardiolipin remodeling, Cell Metab. 20 (2014) 158–171.

[44] S.M. Claypool, Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function, Biochim. Biophys. Acta 1788 (2009) 2059–2068.

[45] M. Lutter, M. Fang, X. Luo, M. Nishijima, X. Xie, X. Wang, Cardiolipin provides specificity for targeting of tBid to mitochondria, Nat. Cell Biol. 2 (2000) 754–761.

[46] S.Y. Choi, P. Huang, G.M. Jenkins, D.C. Chan, J. Schiller, M.A. Frohman, A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis, Nat. Cell Biol. 8 (2006) 1255–1262.

[47] C.T. Chu, J. Ji, R.K. Dagda, J.F. Jiang, Y.Y. Tyurina, A.A. Kapralov, V.A. Tyurin, N. Yanamala, I.H. Shrivastava, D. Mohammadyani, K.Z.Q. Wang, J. Zhu, J. Klein-Seetharaman, K. Balasubramanian, A.A. Amoscato, G. Borisenko, Z. Huang, A.M. Gusdon, A. Cheikhi, E.K. Steer, R. Wang, C. Baty, S. Watkins, I. Bahar, H. Bayir, V.E. Kagan, Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells, Nat. Cell Biol. 15 (2013) 1197–1205.

[48] N. Ishihara, Y. Fujita, T. Oka, K. Mihara, Regulation of mitochondrial morphology through proteolytic cleavage of OPA1, EMBO J. 25 (2006) 2966–2977.

[49] Z. Song, H. Chen, M. Fiket, C. Alexander, D.C. Chan, OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L, J. Cell Biol. 178 (2007) 749–755.

[50] T. Ban, J.A. Heymann, Z. Song, J.E. Hinshaw, D.C. Chan, OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation, Hum. Mol. Genet. 19 (2010) 2113–2122.

[51] V. Del Dotto, P. Mishra, S. Vidoni, M. Fogazza, A. Maresca, L. Caporali, J.M. McCaffery, M. Cappelletti, E. Baruffini, G. Lenaers, D. Chan, M. Rugolo, V. Carelli, C. Zanna, OPA1 isoforms in the hierarchical organization of mitochondrial functions, Cell Rep. 19 (2017) 2557–2571.

[52] H. Lee, S.B. Smith, Y. Yoon, The short variant of the mitochondrial dynamin OPA1 maintains mitochondrial energetics and cristae structure, J. Biol. Chem. 292 (2017) 7115–7130.

[53] Y.W. Lu, S.M. Claypool, Disorders of phospholipid metabolism: an emerging class of mitochondrial disease due to defects in nuclear genes, Front. Genet. 6 (2015) 3.

[54] M. Schlame, Cardiolipin remodeling and the function of tafazzin, Biochim. Biophys. Acta 1831 (2013) 582–588.

[55] J.N. Spelbrink, Functional organization of mammalian mitochondrial DNA in nucleoids: history, recent developments, and future challenges, IUBMB Life 62 (2010) 19–32.

[56] A. Matsumura, J. Higuchi, Y. Watanabe, M. Kato, K. Aoki, S. Akabane, T. Endo, T. Oka, Inactivation of cardiolipin synthase triggers changes in mitochondrial morphology, FEBS let. 592 (2018) 209–218.