Mieap forms membraneless organelles to compartmentalize and facilitate cardiolipin metabolism

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Abstract

Liquid droplets function as membraneless organelles that compartmentalize and facilitate efficient biological reactions. They are formed by proteins with intrinsically disordered regions (IDRs) via liquid–liquid phase separation. Mieap/SPATA18, a p53-inducible protein, participates in suppression of colorectal tumors by promoting mitochondrial quality control. However, the regulatory mechanism involved remains unclear. Here, we report that Mieap is an IDR-containing protein that drives formation of liquid droplets in mitochondria. Mieap liquid droplets specifically phase separate the mitochondrial phospholipid, cardiolipin. Lipidomic analysis of cardiolipin suggests that Mieap promotes enzymatic reactions involved in cardiolipin metabolism, including biosynthesis and remodeling. Accordingly, four cardiolipin biosynthetic enzymes, TAMM41, PGS1, PTPMT1, and CRLS1, and two remodeling enzymes, PLA2G6 and TAZ, are phase-separated by Mieap liquid droplets. Mieap-deficient mice exhibit altered crista structure in mitochondria of various tissues, including brown fat, and tend to become obese. These results suggest that Mieap drives formation of membraneless organelles to compartmentalize and promote cardiolipin metabolism at the inner mitochondrial membrane, thus potentially contributing to mitochondrial quality control.

Introduction

Liquid droplets (LDs) in cells are protein condensates composed of proteins, nucleic acids, and other macromolecular components^{1–3}. LDs are formed by proteins with intrinsically disordered regions (IDRs) via liquid–liquid phase separation (LLPS). Importantly, LDs function as membraneless organelles (MLOs) that compartmentalize and facilitate cellular biological reactions. LDs are not surrounded by lipid bilayers, which enables facile exchange of reactants and products with their surroundings, expediting biological reactions in cells. Furthermore, theoretically, LDs are able to appear and disappear within a cell in response to cellular stress and/or subcellular circumstances, exhibiting spatiotemporal dynamic properties. On the basis of these features, the concept of MLOs is fundamentally different from the long-standing concept of membrane-bound organelles, such as nuclei, lysosomes, mitochondria, and endoplasmic reticulum, stable structures that segregate biomolecules and biological reactions using lipid bilayer membranes. A number of MLOs may exist within a cell, possibly regulating cellular biological reactions and activities, including transcriptional regulation⁴, signal transduction^{5,6}, immunity⁷, centrosome activity⁸, and mitosis⁹. This list is rapidly growing.

Metabolic reactions are believed to be highly organized through spatiotemporal clustering and compartmentalization of sequential enzymes and substrates/intermediates at subcellular sites, which maximizes efficiency of linked reactions¹⁰. Without this mechanism, small, toxic intermediates, derived from metabolic reactions, rapidly diffuse throughout the cytoplasm. Thus, the concept of subcellular compartmentalization of metabolic reactions has been anticipated for a long time. It was initially conceived as a “metabolon,” a structural-functional complex of sequential metabolic enzymes and substrates/intermediates¹¹. The “metabolon” concept predicted that the sequential enzymes and cellular

structural elements form a supramolecular complex for metabolic reactions. In fact, many studies have demonstrated that enzyme clustering in vivo may facilitate sequential enzymatic metabolic reactions¹²⁻¹⁵. Among them, “purinosomes” involved in purine metabolism, were the first demonstration of enzyme clustering, revealed by live-cell imaging of six sequential enzymes for de novo purine synthesis¹⁶. Although MLOs that compartmentalize and expedite metabolic reactions in cells could address regulatory mechanisms for sequential metabolic reactions, there has been no clear evidence for metabolic MLOs until now.

Cardiolipin (CL) is a unique and important phospholipid for the following reasons. [1] It is a phospholipid dimer, with two phosphate residues and four fatty acyl chains¹⁷. [2] Because of this unique structure, it forms a cone shape, contributing to curvature of the lipid membrane and to maintenance of mitochondrial cristae¹⁸. [3] CL is the only phospholipid that is specific to mitochondria and is mainly located at the inner mitochondrial membrane and contact sites¹⁸. [4] CL interacts with many mitochondrial membrane proteins, including electron transport chain complexes involved in oxidative phosphorylation, and the ADP/ATP carrier. Interaction with CL promotes activity of these proteins¹⁸. [5] CL stabilizes the structural assembly and activity of respiratory super-complexes at the inner mitochondrial membrane¹⁹. [6] It interacts with cytochrome *c* at the outer surface of the inner mitochondrial membrane, which supports cellular viability by maintaining stable respiratory ATP production and inducing apoptosis, respectively¹⁹. Therefore, control of CL quality and quantity appears to be important for various mitochondrial functions.

CL metabolic reactions comprise two processes, CL biosynthesis and remodeling. CL metabolic enzymes are characterized and studied in yeast cells, and human orthologs of yeast enzymes have been reported²⁰. In brief, phosphatidic acid (PA) is synthesized in the endoplasmic reticulum and subsequently transported by PRELI (yeast Ups1) via the outer mitochondrial membrane to the inner mitochondrial membrane, as the substrate of CL metabolism^{20,21}. In CL biosynthesis, PA is converted to nascent CL on the matrix face of the inner mitochondrial membrane through sequential enzymatic reactions that produce three intermediates, CDP-diacylglycerol (CDP-DG), phosphatidylglycerophosphate (PGP), and phosphatidylglycerol (PG). These reactions are catalyzed by a series of four enzymes, TAMM41 (yeast Tam41), PGS1 (yeast Pgs1), PTPMT1 (yeast Gep4), and CRLS1 (yeast Crd1) (20,22). The final step of CL metabolic reactions is remodeling of nascent CL to mature CL, which is catalyzed by PLA2G6/iPLA2 β (yeast Cld1) and TAZ (yeast Taz1)^{20,22}. These cascading enzymatic reactions are thought to be continuously executed at the inner mitochondrial membrane. However, how these enzymatic reactions can be continuous and/or how CL metabolic reactions are regulated remain unknown.

Altered CL metabolism and/or deterioration of CL quality and quantity due to CL metabolic enzyme deficiency causes various mitochondrial dysfunctions in eukaryotes ranging from yeast to mammals^{20,23}. Specifically, Barth syndrome is a human disease caused by mutations in the TAZ gene encoding tafazzin, the enzyme responsible for CL remodeling²⁴. Clinical symptoms of Barth syndrome patients include cardiomyopathy, skeletal muscle weakness, neutropenia, and growth retardation. Barth

syndrome models, including lymphoblasts and induced pluripotent stem cells derived from patients, exhibit abnormal crista structures, decreased respiration activity, and increased ROS generation in mitochondria^{25,26}. This implies that substantial regulation of CL metabolic reactions is required to maintain mitochondrial functions and to prevent diseases. However, the regulatory mechanism for CL metabolic reactions is unknown.

Mitochondria-eating protein (Mieap, also designated as SPATA18) was originally identified as a p53-inducible protein. Its mRNA expression is directly regulated by the tumor suppressor, p53, in response to various cellular stresses, including DNA damage²⁷. Mieap expression is lost in nearly 50% of human cancer cell lines due to promoter methylation. Mieap-deficient LS174T colorectal cancer cells generate higher levels of mitochondrial reactive oxygen species (ROS) and synthesize less ATP. Mitochondrial ROS in Mieap-deficient colorectal cancer and gastric cancer cells enhances migration and invasiveness of cancer cells under hypoxic conditions^{28,29}. Mieap deficiency promotes intestinal tumors in ApcMin/+ mice³⁰. Tumors in Mieap-deficient ApcMin/+ mice reveal abnormal mitochondrial morphology, such as large size, round shape, and disordered cristae. Mieap expression is defective in thyroid oncocytic cell tumors, which accumulate abnormal mitochondria in tumor cells³¹. Mieap-regulated mitochondrial quality control is inactivated in tumor tissues of nearly 70% of colorectal cancer and 25% of breast cancer patients^{28,32}. These observations suggest that Mieap suppresses tumors via mitochondrial quality control. However, the mechanism underlying regulation of mitochondrial quality control by Mieap remains unclear.

Here, we report that Mieap is an IDR-containing protein that drives LDs formation in mitochondria. Mieap-induced LDs phase separate the mitochondrial phospholipid, CL. The present findings suggest that Mieap-induced LDs function as MLOs that compartmentalize and promote CL synthesis and remodeling reactions at the inner mitochondrial membrane, leading to stabilization of oxidative phosphorylation and suppression of mitochondrial ROS generation. Thus, we suggest that the Mieap-CL axis is the regulatory mechanism for efficient CL metabolic reactions and/or CL quality and quantity control, forming MLOs that govern mitochondrial quality control by regulating CL synthesis and remodeling. Dysregulation of this pathway leads to mitochondrial dysfunction due to CL alterations, causing a variety of diseases and/or pathologies, including cancer, obesity, and infertility.

Results

Mieap forms LDs in mitochondria

Mieap has been reported to form vacuole-like structures, designated as Mieap-induced vacuoles (MIVs), which were thought to directly assimilate mitochondria³³ in a non-canonical type of mitophagy. To examine MIV formation and “non-canonical mitophagy” in live cells, we constructed a recombinant adenovirus to express EGFP-tagged Mieap and observed cells expressing EGFP-Mieap using confocal live-cell imaging. Multiple spherical green signals (EGFP-Mieap) appeared around the red mitochondrial signals (MitoTracker Red) (Supplementary fig. S1A and Video S1). These oval green signals fused,

enlarged, and eventually coalesced into a few large, spherical structures. In contrast, red mitochondrial signal intensity decreased during this process. These results were consistent with our previous interpretation of non-canonical mitophagy; however, they also indicated that the structures formed by EGFP-Mieap are not the ring-shaped formations, previously reported using immunofluorescence (IF)³³.

Thus, we repeated the IF experiment on structures comprising EGFP-Mieap. EGFP-Mieap reproducibly formed green condensates, while antibodies (anti-Mieap antibody and anti-GFP antibody) produced ring-shaped staining around the green condensates (Supplementary fig. S1B). Thus, we suspected that antibodies are unable to permeabilize EGFP-Mieap condensates.

We performed additional IF using anti-FLAG antibody for the MIV structures, which comprise N-FLAG-Mieap (N-terminal FLAG-tagged Mieap protein) and C-FLAG-Mieap (C-terminal FLAG-tagged Mieap protein). Ring-shaped staining was observed with N-FLAG-Mieap, but not C-FLAG-Mieap (Supplementary fig. S1C), suggesting that Mieap may be positioned with its N-terminal domain facing outward at the periphery of the condensates.

Using transmission electron microscopy (TEM), MIV structures appeared to consist of two phases: a dominant electron-dense phase that stained strongly with OsO₄, and an OsO₄-negative, electron-lucent minor phase (Supplementary fig. S1D). In immunoelectron microscopy (IEM) analysis with anti-Mieap antibody, gold colloid staining indicated that Mieap protein was distributed over the major electron-dense phase (Supplementary fig. S1E). Thus, both fluorescent tagging and antigen-antibody reactions confirmed that Mieap forms protein condensates, a process fundamentally distinct from “mitophagy.” Mieap condensates exhibited spherical or oval shapes, contained bubbles, and showed fusion (Supplementary fig. S1D and Video S2). These characteristics indicate that Mieap condensates have liquid-like properties, suggesting that the structures are LDs³. Therefore, we designate Mieap condensates as Mieap liquid droplets (Mi-LDs).

To clarify the spatial relationship between Mi-LDs and mitochondria, we performed live-cell imaging using cells co-expressing EGFP-Mieap and an outer mitochondrial membrane marker, mApple-TOMM20. In order to thoroughly evaluate the relationship between Mi-LDs and mitochondria, we captured images of nascent Mi-LDs before their enlargement. mApple-TOMM20 entirely surrounded the small Mi-LDs, suggesting that Mieap forms LDs in mitochondria (Fig. 1 A – C, and Supplementary Video S3). Furthermore, our imaging analysis indicated that even enlarged Mi-LDs still exist inside mitochondria (Fig. 1D). Therefore, Mi-LDs are likely the first example of mitochondrial LDs. Considering the efficiency and precision of imaging analyses, we examined enlarged Mi-LDs in the following experiments.

Mieap is an IDR-containing protein with potential to drive LLPS

We performed *in silico* sequence analyses. Mieap orthologs were found in eukaryotes, but not in bacteria, archaea, or viruses (Fig. 2A)³⁴. Hence, the Mieap function evolved in eukaryotes. Moreover, among

eukaryotes, Mieap orthologs were found in metazoans, but not in fungi (Fig. 2A), suggesting that Mieap is beneficial to multicellular organisms.

Using prediction tools³⁵⁻³⁷, we determined that Mieap has four IDRs occurring in two coiled-coil (CC) domains (amino acids 92–311: IDR1 to IDR3) and in the C-terminus (amino acids 499–538: IDR4) (Fig. 2 B – E), suggesting that Mieap can potentially drive LLPS³.

All four IDRs are enriched in arginine (R, positively charged) and serine (S, uncharged polar) (Fig. 2B). Additionally, IDR1 is enriched in aspartic acid (D, negatively charged), and IDR2 is enriched in glutamic acid (E, negatively charged). D and E were mixed with positively charged residues in IDR1 and IDR2, respectively. In contrast, IDR3 and IDR4 formed clusters of positively charged residues, characterized by repeats of R and S (Fig. 2 B – E)³⁸.

Although amino acid sequences of the IDRs are evolutionarily divergent compared to the structured regions, the distribution of IDRs and clusters of positively charged residues in IDR3 and IDR4 are evolutionarily conserved (Fig. 2 C and E, and Supplementary fig. S2)³⁸. In addition, there is an evolutionarily conserved hydrophobic character in the Mieap sequence as a whole, the N-terminal half being hydrophilic and the C-terminal half being hydrophobic (Fig. 2 D and E, and Supplementary fig. S2)³⁹. This implies that Mieap protein may act as a cellular biosurfactant.

Material state and dynamics of Mi-LDs are determined by specific regions of Mieap

To map sites responsible for the physical state and dynamics of Mi-LDs, using a confocal microscope, we examined cells expressing EGFP-Mieap WT (WT) and three deletion-mutant forms, EGFP-Mieap Δ CC, Δ 275, and Δ 496 (Δ CC, Δ 275, and Δ 496) (Fig. 3A).

All deletion mutants formed condensates, exhibiting distinctive morphologies (Fig. 3B and Supplementary Video S4). WT formed spherical or oval condensates that assemble near the nucleus and contain bubbles. In contrast, Δ CC formed lobular condensates without bubbles near the nucleus. Δ 275 formed spherical condensates without bubbles, which are distributed throughout the cytoplasm. Δ 496 formed spherical condensates, which are similar to, but smaller and more numerous than WT condensates.

We further examined the relationship between localization of each mutant and mitochondria by performing live-cell imaging of mutants using MitoTracker Red. WT, Δ CC, and Δ 496 condensates appeared in mitochondria, whereas Δ 275 condensates were detected in the cytoplasm away from mitochondria (Fig. 3C and Supplementary Video S4).

These results suggest that the N-terminal region, containing IDRs (IDR1–3) and spreading over the CC domains, endows Mi-LDs with liquid-like properties. The structured region allows Mi-LDs to reside in

mitochondria, and the C-terminal region, containing IDR4, endows Mi-LDs with the capacity to coalesce.

To investigate protein dynamics in Mi-LDs, we performed fluorescence recovery after photobleaching (FRAP) studies for WT, Δ CC, Δ 275, and Δ 496 condensates. During observations up to 60 s after spot-bleaching, with the bleaching depth being $82.2 \pm 8.1\%$, fluorescence intensity of WT, Δ CC, Δ 275, and Δ 496 condensates recovered to $9.9 \pm 4.0\%$, $4.0 \pm 1.0\%$, $94.3 \pm 4.0\%$, and $17.8 \pm 3.1\%$ of the initial value, respectively (Fig. 3D and Supplementary fig. S3A). Fluorescence recovery of the Δ 275 condensates almost achieved their initial value within the 60-s observation period, indicating the most fluid state.

When we performed less intense laser exposure, which reduced the bleaching depth to $21.2 \pm 4.4\%$, fluorescence recovery increased 60 s after spot-bleaching (WT, $50.4 \pm 8.8\%$; Δ CC, $32.6 \pm 8.8\%$; Δ 275, $94.9 \pm 13.7\%$; Δ 496, $61.5 \pm 10.1\%$) (Supplementary fig. S3B). The fluorescence recovery rate increased when the number of bleached molecules was small, suggesting that availability may be a rate-limiting factor.

We further examined slow fluorescence recovery up to 15 min. WT and Δ 496 condensates showed continuous fluorescence recovery, which reached $80.4 \pm 10.2\%$ and $94.0 \pm 9.5\%$ of the initial value in 15 min, respectively (Fig. 3E). In contrast, Δ CC condensates reached equilibrium at $37.4 \pm 6.3\%$ of their initial value in 15 min (Fig. 3E). These data suggested that WT condensates, as well as Δ 496 condensates, consist mainly of mobile materials, but protein availability from their surroundings is limited. Therefore, FRAP analysis data supported the notion that Mi-LDs exist in mitochondria.

Mieap phase separates mitochondrial CL and CL-binding proteins

To identify molecules targeted for phase separation by Mi-LDs, we screened available fluorescence-tagged mitochondrial proteins and mitochondrial fluorescence probes using confocal live-cell imaging. EGFP-BNIP3, EGFP-NIX, AcGFP1-Mito, DsRed2-Mito, and SYBR Green I (a probe for mitochondrial DNA) were localized at mitochondria, but none of them were incorporated into the Mi-LDs (Fig. 4 A – E, and Supplementary fig. S4 A and B, and Video S5).

However, 10-N-nonyl acridine orange (NAO) and EGFP-cytochrome *c* were specifically incorporated into Mi-LDs (Fig. 4 F and G, and Supplementary fig. S4C and Video S6). NAO targets CL⁴⁰, and cytochrome *c* is a CL-binding protein¹⁹. As described above, positively charged residues are enriched in Mieap IDRs (Fig. 2 B – E). CL carries a net negative charge⁴¹. Sequence data reveal the amphiphilicity of Mieap (Fig. 2 D – E), and Mi-LDs consist of an electron-dense phase that is positive for OsO₄⁴², according to TEM analysis (Supplementary fig. S1D), suggesting that Mi-LDs may contain unsaturated lipids. Therefore, we suspected that CL is a bona-fide target for phase separation by Mi-LDs.

CL forms complexes with many mitochondrial proteins (>60 proteins), such as cytochrome *c*, ATP synthase, and prohibitin complex⁴³. Among them, ATP5F1B and PHB2 were previously listed as

candidates for “Mieap-binding” proteins in our Mieap-interactome data⁴⁴. This prompted us to investigate whether additional CL-binding proteins (ATP5F1B and PHB2) are phase separated by Mi-LDs.

EGFP-ATP5F1B and EGFP-PHB2 localized at mitochondria and then became distributed in Mi-LDs (Fig. 4 H and I, and Supplementary fig. S4 D and E, and Video S6). Although EGFP-cytochrome *c*, EGFP-ATP5F1B, and EGFP-PHB2 co-localized with TagRFP-T-Mieap, EGFP-cytochrome *c*, EGFP-ATP5F1B, and EGFP-PHB2 were excluded from the Mieap-containing major phase and they accumulated in the minor phase (bubbles) in Mi-LDs. These results imply that CL-binding proteins are phase separated, but that they are incorporated into Mi-LDs in association with CL.

To determine whether Mieap binds directly to CL, we performed a fat blot assay⁴⁵, in which binding of GST-Mieap to CL, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) was evaluated on lipid-dotted membranes. GST-Mieap bound to CL, but not to PC or PE (Fig. 4J).

Mi-LDs compartmentalize and facilitate CL metabolic reactions

We performed mass spectrometric analyses of CL in A549 cells with and without exogenous Mieap expression. The total amount of CL per cell was higher in Mieap-expressing cells than in cells without Mieap expression (Fig. 5A). Broad CL species showed higher absolute values in cells with Mieap expression than in cells without it (Fig. 5B and Supplementary Data S1). In contrast, most of the relative amounts of each CL species did not change. However, Mieap specifically increased the proportions of CL72:4, CL72:5, CL72:6, CL70:5, CL70:6, CL68:5, and CL68:6 (Fig. 5B). These results suggest that Mieap is involved in CL metabolism. Therefore, we speculated that Mi-LDs may function as MLOs to compartmentalize and facilitate CL metabolic reactions. To validate this hypothesis, we examined whether Mi-LDs phase-separate enzymes sequentially involved in CL metabolism (Fig. 5C).

Thus, we examined involvement of the following EGFP-tagged enzymes required for CL metabolism in Mi-LDs by performing confocal live-cell imaging: EGFP-TAMM41, EGFP-PGS1, EGFP-PTPMT1, and EGFP-CRLS1 (involved in CL biosynthesis); EGFP-PLA2G6 (related to CL hydrolysis by phospholipase A₂ activity); and EGFP-TAZ (involved in CL remodeling). All of these enzymes localized at mitochondria and were subsequently incorporated into Mi-LDs (Fig. 5 D – I, and Supplementary fig. S5 A – F, and Video S7).

Interestingly, all of these CL metabolic enzymes were largely concentrated in the internal bubbles (the electron-lucent minor phase) in Mi-LDs (Fig. 5 D – I, and Supplementary Video S7). Additionally, unlike CL-binding proteins, most enzymes were localized across the entire circumference of Mi-LDs, as indicated by line-scan analysis (Fig. 5 D – I). These results suggest that the external edge of Mi-LDs faces the inner mitochondrial membrane and that enzymes are incorporated in bubbles at the interface between the inner mitochondrial membrane and external edges of Mi-LDs.

We further examined three additional mitochondrial proteins, PRELI (EGFP-PRELI), mitochondrial protease LONP1 (EGFP-LONP1), and mitochondrial CL hydrolase/mitochondrial phospholipase (MitoPLD)/phospholipase D6 (EGFP-PLD6). PRELI is a mitochondrial carrier of PA for CL production (21). LONP1 is a AAA mitochondrial protease⁴⁶. PLD6 hydrolyzes CL to generate PA at the outer mitochondrial membrane⁴⁷. EGFP-PRELI was incorporated into Mi-LDs (Fig. 5J, and Supplementary fig. S5G and Video S7), suggesting that Mi-LDs can also be supplied with PA as a substrate for CL synthesis (Fig. 5C). Both EGFP-LONP1 and EGFP-PLD6 were located at mitochondria, but neither was distributed in Mi-LDs (Fig. 5K and L, and Supplementary fig. S5H and I, and Video S7).

Mieap functions in mitochondrial quality control via regulation of CL metabolism

CL alterations cause mitochondrial dysfunction^{20,23}. Mieap is thought to be involved in mitochondrial quality control^{27,28,30,33}. Therefore, we hypothesized that Mieap contributes to mitochondrial quality control by regulating CL metabolism. To test this hypothesis, we evaluated mitochondrial status relative to CL integrity in Mieap-deficient cells and mice.

First, we examined respiration rate, mitochondrial ATP production rate, crista morphology, and ROS level, in control and Mieap-KD LS174T cells under physiological conditions, all of which reflect CL integrity. Flux analysis indicated that respiration activity and ATP production rate of Mieap-KD cells were significantly lower than those of control cells (Fig. 6A and B). TEM analysis revealed that cristae of Mieap-KD cells decreased, and their morphology became unclear and irregular, compared to that of control cells (Fig. 6C and D). ROS levels increased in Mieap-KD cells (Fig. 6E). Consistently, the total amount of CL in control cells was higher than in Mieap-KD cells (Fig. 6F), and control cells showed higher absolute values for broad CL species than Mieap-KD cells (Fig. 6G and Supplementary Data S1). Furthermore, physiological Mieap specifically increased the relative values of CL72:4, CL72:5, CL70:4, CL70:5, CL68:3, and CL68:4 in LS174T cells (Fig. 6G).

Second, we analyzed crista morphology in kidney and liver tissues of Mieap-knockout (KO) mice by performing TEM analysis. In Mieap^{-/-} kidney mitochondria, irregularly dilated lamellar structures without distinct OsO₄ staining were observed (Fig. 6H). A decrease in normal crista structure was a common characteristic of Mieap^{-/-} mitochondria in the kidney and liver (Fig. 6I – K). Accordingly, Mieap^{+/+} kidney and liver mitochondria exhibited higher absolute values for broad CL species than Mieap^{-/-} kidney and liver mitochondria (Fig. 6L and M).

Finally, we performed a large-scale cross-sectional observation of a total of 1353 Mieap^{+/+}, Mieap^{+/-}, and Mieap^{-/-} mice to identify long-term consequences of Mieap deficiency. Average body weights of Mieap^{+/+} and Mieap^{-/-} mice were higher than those of Mieap^{+/-} mice throughout their lives (Fig. 7A and B). Differences were prominent during middle and old age, from 40 to 104 weeks (Fig. 7C) (mean value ± SE; Mieap^{+/+} 33.154±0.410 g [n=162], Mieap^{+/-} 34.099±0.289 g [n=303], Mieap^{-/-} 35.090±0.415 g [n=175]),

but particularly during middle age, from 53 to 62 weeks (Fig. 7D) (mean value $\pm$ SE; *Mieap*^{+/+} 31.048 $\pm$ 0.64 g [n=49], *Mieap*^{+/-} 33.378 $\pm$ 0.517 g [n=115], *Mieap*^{-/-} 34.579 $\pm$ 0.660 g [n=68]). Reduced mitochondrial respiratory activity in adipose tissues has been suggested as a factor contributing to obesity⁴⁸. Brown fat tissue (BAT) is essential to heat production via both its respiratory activity, which generates a proton gradient, and uncoupling protein 1 (UCP1)-mediated proton leakage across the mitochondrial inner membrane^{49,50}. Therefore, BAT mitigates obesity. Importantly, as a CL-binding protein, activity of UCP1 is stabilized by CL⁵¹. Thus, we examined the status of mitochondrial cristae in BAT of *Mieap*^{-/-} mice. We confirmed that normal crista structure was significantly decreased in *Mieap*-deficient BAT (Fig. 7 E and F), suggesting that obesity may be a long-term consequence of mitochondrial dysfunction due to CL alteration in tissues of *Mieap*-deficient mice, including BAT.

Therefore, we concluded that all these phenotypes in *Mieap*-deficient models corroborate our hypothesis that *Mieap* serves a physiological function in mitochondrial quality control by regulating CL metabolism.

Discussion

Although the importance of multi-enzyme complexes in metabolic enzyme reactions has been recognized, it remains unclear how this complex of enzymes efficiently and safely enables sequential enzymatic reactions by preventing diffusion of intermediates. A recent report suggested that concentration of multiple enzymes and substrates/intermediates in a restricted space could mediate efficient sequential enzymatic reactions by preventing diffusion of intermediates⁵². In that model, multiple copies of upstream and downstream enzymes involved in sequential enzymatic reactions are assembled into a single cluster, called an “agglomerate.” According to this model, “once an upstream enzyme produces an intermediate; although the probability of the intermediate being processed by any individual downstream enzymes is low, the probability that the intermediate will be processed by one of the many downstream enzymes in the ‘agglomerate’ can be high”⁵². Therefore, based on this model, molecular crowding of enzymes, substrates, and intermediates in a restricted space could enable efficient sequential enzymatic reactions mediated by multiple enzymes.

The “agglomerate” concept is promising, but an important question remains: how can so many diverse molecules, including multiple enzymes, substrates, and intermediates, be gathered, concentrated, and compartmentalized within a single restricted space? What drives formation of the “agglomerate”? We speculate that LDs could organize agglomerates as metabolic LDs⁵³. Accumulating evidence suggests that LDs can function as MLOs, which promote biochemical reactions by concentrating and compartmentalizing enzymes and substrates in cells⁵⁴⁻⁵⁷. Since LDs are not surrounded by lipid bilayers, theoretically, they exhibit spatiotemporal dynamic properties within a cell in response to cellular stress and/or subcellular circumstances. More importantly, while LDs contain hundreds of molecules, a few scaffold proteins can drive formation of these MLOs¹. If there are proteins that can organize metabolic LDs as scaffolds⁵⁶, these agglomerates could enable efficient metabolic reactions.

In the present study, we obtained the following evidence supporting our hypothesis that Mi-LDs may regulate CL metabolism: [1] Mieap drives formation of LDs (Mi-LDs) in mitochondria. [2] Mi-LDs contain CL. [3] Mi-LDs contain all four sequential enzymes for CL biosynthesis (TAMM41, PGS1, PTPMT1, and CRLS1). [4] Mi-LDs contain two enzymes for CL remodeling (PLA2G6 and TAZ). [5] Mi-LDs contain at least three CL-binding proteins localized at the inner mitochondrial membrane (cytochrome *c*, PHB2, and ATP5F1B). [6] The presence or absence of Mieap protein is closely related to an increase or decrease in various species of CL, respectively. [7] The presence of Mieap protein specifically increases the proportion of several species of CLs. [8] Mieap deficiency is related to changes in crista structure and CL metabolism *in vivo*. This evidence suggests that Mieap, is a scaffold protein that drives formation of metabolic LDs to compartmentalize and concentrate enzymes, substrates, and intermediates that are involved in CL biosynthesis and remodeling, leading to molecular crowding within the Mi-LDs that promotes efficient catalysis of CL metabolic reactions.

In Mi-LDs, CL and Mieap occur in the major phase (electron-dense region in TEM), whereas all CL biosynthesis and remodeling enzymes, including TAMM41, PGS1, PTPMT1, CRLS1, PLA2G6, and TAZ, are predominantly segregated into the minor phase (electron-lucent region in TEM). This result suggests that substrates, intermediates, and products for CL metabolism do not reside in the same phase as their catalytic enzymes. Why don't substrates and enzymes occur in the same phase of the Mi-LD? We offer two possible explanations for this observation. First, since substrates/intermediates in Mi-LDs are lipids, the water-soluble enzymes cannot mix with lipids. Second, crowding of enzymes and substrates in a single phase might result in substrate inhibition⁵⁸. A recent study demonstrated that sequestration of enzymes to a membraneless compartment that is away from, but adjacent to substrates, can accelerate reactions much faster than when the enzymes are mixed with the substrates in the same compartment⁵⁸. This implies that the separation of enzymes from their substrates via LLPS could facilitate enzymatic reactions. In this case, the interface between the enzyme and substrate phases would be the site of the reaction.

Mieap may serve as a biosurfactant and a binding protein for CL. Both its biosurfactant activity and its binding to CL could be critical for Mi-LD formation in mitochondria. Moreover, we hypothesize the following model for sequential CL-metabolic reactions promoted by Mieap (Supplementary fig. S6). Mieap may stably interact with the substrate (PA) via its C-terminal, hydrophobic, structured region, which exhibits a specific, strong interaction. On the other hand, Mieap weakly and transiently interacts with CL-metabolizing enzymes via its N-terminal hydrophilic region, which exhibits multiple interactions. In this model, once Mieap grabs the substrate via its C-terminal region, Mieap enables sequential CL metabolic reactions by transiently interacting with the enzyme corresponding to the substrate at the interface between the major (lipids) and minor phases (enzymes), and then changing enzymes until mature CL is produced. Molecular crowding in Mi-LDs, phase separation of enzymes and substrates, interfacial catalysis, and biosurfactant activity could foster highly efficient sequential enzymatic reactions for CL metabolism. We suggest that Mi-LDs may be the first known metabolic MLOs to regulate CL synthesis and remodeling. Further investigation of this hypothesis is required.

Significant evidence is now emerging, suggesting a critical role of Mieap in mitochondrial quality control in response to cellular stresses. Under hypoxic conditions, Mieap-deficient gastric cancer cells exhibit abnormal mitochondrial structure, enhanced migration, and invasion via increased mitochondrial ROS generation²⁹. Mieap expression is defective in oncocytic cell tumors of the thyroid, suggesting a causative role in accumulation of abnormal mitochondria in tumor cells³¹. Oncocytic tumors are characterized by accumulation of abnormal mitochondria in tumor cells, but the underlying mechanism remains unclear. Recently, Mieap/Spata18 was reported to participate in DNA-damage-induced mitophagy⁵⁹. Results in these studies are largely consistent with our previous observations^{27,33}. However, all observations from these studies, as well as our previous work can be explained by the idea that Mieap influences mitochondrial quality control by regulating CL metabolism. The only unaddressed question is whether lysosomes/lysosomal proteins are involved in the function of Mieap. In response, we have now confirmed that EGFP-cathepsin D is localized at mitochondria and then incorporated into Mi-LDs (unpublished data). Although resolving this question will be challenging, we strongly suggest that the mechanism underlying these observations is executed by regulation of CL metabolism by Mieap

In the present study, we demonstrated that Mieap-deficient LS174T cells exhibited altered CL metabolism, decreased respiration activity, increased ROS levels, and abnormal crista structure, all of which are consistent with phenotypes induced by CL alteration. We also found that Mieap-deficient mice exhibit decreased numbers and morphological abnormalities of mitochondrial crista structure and altered CL metabolism in kidney and liver tissues. Furthermore, our body weight analysis of Mieap^{+/+}, Mieap^{+/-}, and Mieap^{-/-} mice clarified an obesity-related trend in Mieap-deficient mice, which is likely attributed to mitochondrial dysfunction in various tissues, including BAT. Therefore, we assume that all these phenotypes in Mieap-deficient cells and mice reflect mitochondrial dysfunctions related to abnormal CL metabolism. So far, autophagy and proteostasis are two major mechanisms in mitochondrial quality control⁶⁰. In addition to these, we suggest that the Mieap-regulated pathway is the third mechanism for mitochondrial quality control, in which Mieap maintains the integrity of mitochondria by regulating CL metabolism. Mieap maintains mitochondrial quality through homeostasis of the inner mitochondrial membrane by regulating CL metabolism, stabilizing oxidative phosphorylation, and suppressing mitochondrial ROS generation (Fig. 7G).

Previously, we reported that although Mieap-deficient mice did not suffer from intestinal dysfunction, Mieap-deficient Apc^{Min/+} mice exhibited remarkable intestinal tumor generation and malignant transformation, compared to Mieap-WT Apc^{Min/+} mice³⁰. Furthermore, mitochondria in Mieap-deficient tumors revealed abnormal morphology, including fewer cristae and enlarged, spherical mitochondria. These results support the role of Mieap in mitochondrial quality control through control of CL metabolism in response to oncogenic stress. In addition to Apc^{Min/+} mice, we observed a similar effect in a Mieap-deficient gastric cancer mouse model (unpublished data), using the *K19-Wnt1/C2mE* transgenic mice⁶¹. Therefore, Mieap-regulated mitochondrial quality control could be critical in tumor suppression by promoting CL metabolism, which leads to upregulation of respiratory activity and downregulation of mitochondrial ROS generation. Considering the role of Mieap in p53 function, we suggest that Mieap

could act as a spatiotemporal and dynamic regulator/modulator in CL metabolism to suppress tumor initiation and progression. Recently, we found that Mieap-deficient sperm in Mieap-KO mice cause *in vitro* infertility due to mitochondrial ROS elevation and impaired sperm motility (unpublished data). Therefore, in addition to cancer and obesity, it is possible that alterations of Mieap-regulated mitochondrial quality control also promote infertility (Fig. 7G).

A recent study reported that PGS1, PTPMT1, and CRLS1 form a large mitochondrial CL synthesis complex in human cells, with a molecular mass of nearly 700-800 kDa⁶². This large CL synthesis complex includes multiple CL-binding proteins. These observations are very similar to our results that Mi-LDs contain all CL synthesis enzymes and several CL-binding proteins. However, IF experiments on PGS1 or CRLS1 in that study indicated that neither PGS1 nor CRLS1 showed visible Mi-LD like structures in mitochondria⁶². Our results from live-cell imaging of EGFP-fused CL synthesis enzymes, including PGS1 and CRLS1, also showed that none of the EGFP-tagged CL synthesis enzymes exhibit visible Mi-LD-like structures. Therefore, we suggest that under physiological conditions, the minute size of Mi-LDs or possibly layered, membraneless organelles induced by Mieap may compartmentalize and facilitate CL metabolic reactions *in vivo*, efficiently maintaining enzymatic reactions. Further investigation is needed to clarify the real picture of physiological membraneless organelles involved in CL metabolism.

Materials And Methods

Cell lines

The following cell lines were purchased from the American Type Culture Collection: A549 (tissue, lung cancer; gender, male), U373MG (tissue, glioblastoma; gender, male), LS174T (tissue, colon cancer; gender, female), and 293 (tissue, embryonic kidney). A549 cells were cultured in RPMI 1640 (Sigma). LS174T and 293 cells were cultured in DMEM (Sigma). All media were supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified chamber with 5% CO₂. These cell lines have not been authenticated.

Mice

Animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation (approved protocol No. T17-043), and the experiments were conducted in accordance with Guidelines for Animal Experiments of the National Cancer Center. C57BL/6J WT mice were obtained from CLEA Japan (Tokyo, Japan). Mieap-knockout (Mieap^{-/-}) mice were generated using the Cre/loxP recombination system, as previously reported³⁰. Briefly, floxed and trapped alleles were generated using a single construct bearing a gene-trap cassette doubly flanked by LoxP and FRT, located between exons 5 and 8 of the mouse Mieap gene, which is located on chromosome 5. Mieap homozygous (Mieap^{-/-}) deficient

mice were generated by mating breeding pairs of Mieap heterozygous (Mieap^{+/-}) mice. All mice were housed at 22 ± 2°C with a 12 h light/dark cycle with free access to food, CE-2 (CLEA Japan), and water.

Establishment of KD cell lines

We established a Mieap-KD cell line using LS174T, as previously described²⁷. Mieap expression was inhibited in the cell line by retroviral expression of short-hairpin RNA (shRNA) against the Mieap sequence. We also established LS174T-cont cells using a retroviral vector with a target sequence for EGFP, or an empty retroviral vector, and A549-cont cells using an empty retroviral vector.

Plasmid construction

Constructs containing Mieap

For construction of the plasmid containing N-terminal EGFP-tagged Mieap, the nucleotide sequence of Mieap was PCR-amplified using primers N-EGFP-Mieap-F and N-EGFP-Mieap-R. PCR products were digested with Kpn I and ligated into pEGFP-C1 (Clontech) cut with the same enzyme. For construction of the plasmid containing C-terminal EGFP-tagged Mieap, the nucleotide sequence of Mieap, excluding the stop codon, was PCR-amplified using the primers C-EGFP-Mieap-F and C-EGFP-Mieap-R. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Inserted products were excised using Hind III restriction sites, and ligated into pEGFP-N1 (Clontech), cut with the same enzyme. N-terminal EGFP-tagged Mieap was used as EGFP-Mieap, except for Fig. 1A – 1D and Supplementary Video S3 where the C-terminal EGFP-tagged Mieap was used.

The plasmid containing N-FLAG-Mieap (pN-FLAG-Mieap) was constructed as follows. The nucleotide sequence of Mieap was PCR-amplified using the primers, N-FLAG-Mieap-F and N-FLAG-Mieap-R. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Inserted products were excised using the Kpn I restriction sites and ligated into pre-digested pcDNA3.1 (+) (Thermo Fisher Scientific) cut with the same enzyme. The nucleotide sequence of Mieap was excised from the plasmid using the Hind III and Xho I restriction sites, and ligated into pre-digested pCMV-Tag2A (Agilent) cut with the same enzyme.

The plasmid containing C-FLAG-Mieap (pC-FLAG-Mieap) was constructed as follows. The nucleotide sequence of Mieap, excluding the stop codon, was PCR-amplified using the primers, C-EGFP-Mieap-F and C-EGFP-Mieap-R, the same primers used for construction of the plasmid containing C-terminal EGFP-tagged Mieap. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Inserted products were excised using the Hind III restriction site and ligated into pre-digested p3xFLAG-CMV-14 (Sigma Aldrich) cut with the same enzyme.

Prior to construction of the plasmid containing EGFP-Mieap deletion mutants, point mutations in Bgl II, Sac I, EcoR I, and Pst I restriction sites of the multiple cloning site of pEGFP-Mieap were introduced using QuikChange Site-Directed Mutagenesis Kits (Agilent) with primers Mut-F1, Mut-R1, Mut-F2 and Mut-R2, which were confirmed by DNA sequencing.

For construction of the plasmid containing EGFP-Mieap Δ CC (pEGFP-Mieap Δ CC), the nucleotide sequence of pEGFP-Mieap between two Pst I restriction sites was deleted by digestion with Pst I. The remainder was self-ligated, additionally deleting c.810C using the QuikChange Site-Directed Mutagenesis Kit (Agilent) with primers Mut-F3 and Mut-R3 to make the deletion mutation in-frame.

For construction of the plasmid containing EGFP-Mieap Δ 275 (pEGFP-Mieap Δ 275), the nucleotide sequence of pEGFP-Mieap between the Bgl II and Sma I restriction sites was deleted by digestion using Bgl II and Sma I. After blunting with T4 DNA polymerase (Thermo Fisher Scientific), the remainder was self-ligated.

For construction of the plasmid containing EGFP-Mieap Δ 496 (pEGFP-Mieap Δ 496), the nucleotide sequence of pEGFP-Mieap between the EcoR I and Kpn I restriction sites was deleted by digestion using EcoR I and Kpn I. After blunting with T4 DNA polymerase (Thermo Fisher Scientific), the remainder was self-ligated.

For construction of the plasmid containing TagRFP-T-Mieap (pTagRFP-T-Mieap), the nucleotide sequence of pEGFP-Mieap between the Nhe I and Xho I restriction sites containing EGFP was replaced with nucleotide sequence of pTagRFP-TEEA1 (Addgene #42635) between the Nhe I and Xho I restriction sites containing TagRFP-T, by digestion using Nhe I and Xho I.

For construction of the plasmid containing GST-Mieap (pGST-Mieap), the nucleotide sequence of Mieap (amino acids 99-298) was PCR-amplified using the primers, GST-Mieap-F and GST-Mieap-R. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Products were digested with EcoR I and Xho I, and ligated into pGEX5X-2 (Cytiva).

Other constructs

For construction of the plasmid containing EGFP-BNIP3 (pEGFP-BNIP3), a plasmid containing FLAG-BNIP3 (pCMV-Tag2B-BNIP3) was constructed in advance. For construction of the pCMV-Tag2B-BNIP3, the nucleotide sequence of BNIP3 was PCR-amplified using the primers, BNIP3-F and BNIP3-R. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Inserted products were digested with EcoR I and Xho I, and ligated into the pre-digested pCMV-Tag2B (Agilent) cut with the same enzyme. The nucleotide sequence of pCMV-Tag2B-BNIP3 was digested at the EcoR I and Xho I restriction sites, and subsequently blunted with T4 DNA polymerase (Thermo Fisher Scientific). pEGFP-C1 (Clontech) was digested with Bgl II, blunted with T4 DNA polymerase, self-ligated, digested with EcoR I and Sma I, and ligated with the fragment of pCMV-Tag2B-BNIP3.

For construction of the plasmid containing EGFP-NIX (pEGFP-NIX), a plasmid containing FLAG-NIX (pCMV-Tag2B-NIX) was constructed in advance. For construction of the pCMV-Tag2B-NIX, the nucleotide sequence of NIX was PCR-amplified using the primers, NIX-F and NIX-R. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Inserted products were digested with EcoR I and Xho I, and ligated into pre-digested pCMV-Tag2B (Agilent) cut with the same enzyme. The nucleotide sequence of pCMV-Tag2B-NIX was digested at the EcoR I and Xho I restriction sites, and subsequently blunted with T4 DNA polymerase (Thermo Fisher Scientific). pEGFP-C1 (Clontech) was digested with Bgl II, blunted with T4 DNA polymerase, self-ligated, digested with EcoR I and Sma I, and ligated with the fragment of pCMV-Tag2B-NIX.

The plasmid containing EGFP-cytochrome *c* (pEGFP-cytochrome *c*) was constructed as follows. The nucleotide sequence of cytochrome *c* was PCR-amplified using the primers (Cytochrome *c*-F and Cytochrome *c*-R) as reported by Goldstein et al.⁶³. PCR products were ligated into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific) and sequenced. Inserted products were excised using the EcoR I and BamH I restriction sites, and ligated into pEGFP-C1 (Clontech) cut with the same enzymes.

For construction of the plasmid backbone containing EGFP (pN-EGFP), the nucleotide sequence of EGFP, excluding the stop codon, was PCR-amplified using the primers, N-EGFP-F and N-EGFP-R. PCR products were digested with Hind III and BamH I, and ligated into pcDNA3.1 (+) (Thermo Fisher Scientific) cut with the same enzymes.

For construction of the plasmid backbone containing EGFP (pC-EGFP), the nucleotide sequence of EGFP was PCR-amplified using the primers, C-EGFP-F and C-EGFP-R. PCR products were digested with BamH I and Not I, and ligated into pcDNA3.1 (+) (Thermo Fisher Scientific) cut with the same enzymes.

For construction of the plasmid containing EGFP-ATP5F1B (pEGFP-ATP5F1B), the nucleotide sequence of ATP5F1B, excluding the stop codon, was PCR-amplified using the primers, ATP5F1B-F1, ATP5F1B-R1, ATP5F1B-F2, and ATP5F1B-R2. PCR products were digested with Hind III and BamH I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-PHB2 (pEGFP-PHB2), the nucleotide sequence of PHB2, excluding the stop codon, was PCR-amplified using the primers, PHB2-F and PHB2-R. PCR products were digested with Nhe I and Kpn I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-TAMM41 (pEGFP-TAMM41), the nucleotide sequence of TAMM41, excluding the stop codon, was PCR-amplified using the primers, TAMM41-F and TAMM41-R. PCR products were digested with Nhe I and BamH I, and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-PGS1 (pEGFP-PGS1), the nucleotide sequence of PGS1, excluding stop codon, was PCR-amplified using the primers, PGS1-F and PGS1-R. PCR products were digested with Hind III and BamH I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-PTPMT1 (pEGFP-PTPMT1), the nucleotide sequence of PTPMT1, excluding the stop codon, was PCR-amplified using the primers, PTPMT1-F and PTPMT1-R. PCR products were digested with Nhe I and Hind III and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-CRLS1 (pEGFP-CRLS1), the nucleotide sequence of CRLS1 was PCR-amplified using the primers, CRLS1-F and CRLS1-R. PCR products were digested with BamH I and Not I and ligated into pN-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-PLA2G6 (pEGFP-PLA2G6), the nucleotide sequence of PLA2G6, excluding the stop codon, was PCR-amplified using the primers, PLA2G6-F and PLA2G6-R. PCR products were digested with Nhe I and Kpn I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-TAZ (pEGFP-TAZ), the nucleotide sequence of TAZ, excluding the stop codon, was PCR-amplified using the primers, TAZ-F and TAZ-R. PCR products were digested with Nhe I and Kpn I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-PRELI (pEGFP-PRELI), the nucleotide sequence of PRELI, excluding the stop codon, was PCR-amplified using the primers, PRELI-F and PRELI-R. PCR products were digested with Nhe I and BamH I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-LONP1 (pEGFP-LONP1), the nucleotide sequence of LONP1, excluding the stop codon, was PCR-amplified using the primers, LONP1-F and LONP1-R. PCR products were digested with Hind III and BamH I and ligated into pC-EGFP cut with the same enzymes.

For construction of the plasmid containing EGFP-PLD6 (pEGFP-PLD6), the nucleotide sequence of PLD6, excluding the stop codon, was PCR-amplified using the primers, PLD6-F and PLD6-R. PCR products were digested with Nhe I and Kpn I and ligated into pC-EGFP cut with the same enzymes.

Transfection

For transfection, cells were seeded (2×10^5 cells/dish) in 35-mm glass bottom dishes. Plasmids (2 μ g /dish) were transfected using FuGENE6 transfection reagent (Promega), according to the manufacturer's instructions.

Recombinant adenovirus construction

Ad-Mieap was derived from viruses as previously reported^{27,33,64}. Replication-deficient recombinant viruses Ad-EGFP-Mieap, Ad-N-FLAG-Mieap, Ad-C-FLAG-Mieap, Ad-EGFP-Mieap Δ CC (Δ 104-270), Ad-EGFP-Mieap Δ 275, Ad-EGFP-Mieap Δ 496, Ad-TagRFP-T-Mieap, Ad-mApple-TOMM20 derived from mApple-TOMM20-N-10 (Addgene #54955), Ad-EGFP-BNIP3, Ad-EGFP-NIX, Ad-AcGFP1-Mito, Ad-DsRed2-Mito, and

Ad-EGFP-cytochrome *c* were generated from the corresponding plasmid vectors and purified as described previously⁶⁵. Briefly, DNA fragments obtained by restriction of each plasmid vector were blunted using T4 DNA polymerase, ligated into the *Sma*I site of the cosmid, pAxCawtit (Takara), which contains the CAG promoter and the entire genome of type 5 adenovirus, except the E1 and E3 regions. Recombinant adenoviruses were generated by *in vitro* homologous recombination in the 293 cell line with the cDNA-inserted pAxCawtit and the adenovirus DNA terminal–protein complex. Viruses were propagated in the 293 cell line and purified by two rounds of CsCl density centrifugation. Viral titers were determined with a limiting dilution bioassay using 293 cells.

Adenoviral infection

Infection of cell lines was carried out by adding viral solutions to cell monolayers, incubating them at 37°C for 120 min with brief agitation every 20 min. This was followed by addition of culture medium and return of the infected cells to the 37°C incubator.

Immunocytochemistry

For immunocytochemistry, cells were grown on 8-well chamber slides ($1-4 \times 10^4$ cells/well) at 37°C in conventional culture medium, and fixed in paraformaldehyde (Figure S1B, 2%; Figure S1C, 4%) for 15 min at room temperature. Slides were incubated with Triton X-100 (Figure S1B, 0.1% for 2 min; Figure S1C, 0.5% for 10 min), and washed 3x with phosphate-buffered saline (PBS) at room temperature. Cells were blocked with 3% bovine serum albumin (BSA) in PBS (Supplementary Figure S1B, for 3 h; Supplementary Figure S1C, for 2 h), and sequentially incubated with rabbit polyclonal anti-Mieap antibody (1:200), mouse monoclonal anti-GFP antibody (1:200), or mouse monoclonal anti-FLAG antibody (1:1000) for 2 h at room temperature. After washing 3x with PBS, slides were incubated with Alexa Fluor 546 goat anti-rabbit IgG antibody (1:200) or Alexa Fluor 546 goat anti-mouse IgG antibody (1:200) at room temperature (Supplementary Figure S1B, for 2 h; Supplementary Figure S1C, for 1 h). Slides were washed 3x with PBS. Then they were mounted with VECTASHIELD H-1000 (Vector Laboratories) and observed using a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus).

Transmission electron microscopy (TEM)

A549-cont and U373MG cells (4×10^4 cells/24-well plate) were infected with Ad-Mieap. On day 1 after infection, cells were fixed in phosphate buffered 2.5% glutaraldehyde and subsequently post-fixed in 1% OsO_4 at 4°C for 2 h. Then, specimens were dehydrated in a graded ethanol series and embedded in epoxy resin. Ultrathin sections (75 nm) were cut with an ultramicrotome. Ultrathin sections stained with uranyl

acetate and lead staining solution were observed on a transmission electron microscope H-7500 (Hitachi) at 80 kV.

LS174T (control and Mieap-KD) cells cultured under normal conditions were also processed for TEM as mentioned above, with the following modifications. 2% glutaraldehyde was used for prefixation. 2% OsO₄ was used instead for post-fixation. 80-90-nm ultrathin sections were cut and observed on a transmission electron microscope H-7600 (Hitachi) at 100 kV.

Kidney and liver specimens were collected from an 18-week-old WT mouse and a 16-week-old Mieap^{-/-} male mouse. BAT specimens were collected from a 40-week-old WT and a 40-week-old Mieap^{-/-} male mouse. Specimens cut into approximately 3 × 3 × 3 mm³ were also processed for TEM and processed in the same fashion as the aforementioned A549-cont cells. However, a mixture of 2% paraformaldehyde and 2% glutaraldehyde was used for prefixation.

Post-embedding immunoelectron microscopy

A549-cont cells (2×10⁵ cells/35-mm glass bottom dish) were infected with Ad-Mieap. On day 1 after infection, cells were fixed with 4% paraformaldehyde and 0.025% glutaraldehyde in 0.1 M PBS (pH 7.4) for 1 h at 4°C. After fixation, cells were washed with 0.1 M PBS (pH 7.4) for 16 h at 4°C, dehydrated in a graded ethanol series, and infiltrated with LR White resin. Polymerization was performed in TAAB embedding capsules (TAAB) inverted on glass-bottom dishes for 3h at 60°C. Ultrathin sections (75 nm) were collected on nickel grids. After blocking with 3% BSA in PBS for 1 h, sections were incubated with anti-Mieap antibody (1:200) diluted in PBS with 0.05% Triton X-100 for 2h at RT. Sections were washed 8x with 0.15% glycine in PBS, and incubated with goat anti-rabbit IgG 10-nm gold antibody (1:50) diluted in PBS with 0.05% Triton X-100 for 2h at RT. Sections were washed 8x in PBS and fixed with 1% glutaraldehyde in PBS for 5 min. Sections were washed 8x in distilled water. Grids were embedded in a mixture containing 2.7% polyvinyl alcohol and 0.3% uranyl acetate. Sections on grids were observed on a transmission electron microscope H-7500 (Hitachi) at 75 kV.

Amino acid sequence analyses of Mieap protein

We analyzed the phylogenetic spread of Mieap orthologs using OrthoDB v10 (<https://www.orthodb.org>)³⁴. Multiple sequence alignment for Mieap orthologs was performed using Genetyx ver. 10. Prediction of IDRs in the amino acid sequence of Mieap was done using VL3-BA³⁶ on the PONDR server (<http://www.pondr.com/>) and collated with meta-prediction of IDRs using DisMeta (<http://www-nmr.cabm.rutgers.edu/bioinformatics/disorder/>)³⁵. Prediction of coiled-coil regions was done using COILS (https://embnet.vital-it.ch/software/COILS_form.html)³⁷. Hydrophobicity of Mieap was analyzed according to the Kyte-Doolittle index³⁹ using ProtScale (<https://web.expasy.org/protscale/>)⁶⁶.

The linear net charge per residue of Mieap was analyzed using CIDER (<http://pappulab.wustl.edu/CIDER/>)³⁸.

Analyses of confocal microscopy image data

Throughout the study, confocal microscopy images were taken with a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus). For validation of the spatial relationship between MLDs and mApple-TOMM20, additional images were taken using a SpinSR10 spinning disk confocal super resolution microscope (Olympus). For Z-stack and time-lapse imaging, a montage of differential interference contrast (DIC) and fluorescence images was created using MetaMorph ver. 7.8 (Molecular Devices). 3D reconstruction was performed using cellSens Imaging Software (Olympus). Line-scan profiles were acquired using MetaMorph ver. 7.8 (Molecular Devices).

FRAP experiments

EGFP-Mieap, EGFP-Mieap Δ CC, EGFP-Mieap Δ 275, and EGFP-Mieap Δ 496 were expressed in A549-cont cells to generate condensates by infection with Ad-EGFP-Mieap, Ad-EGFP-Mieap Δ CC, Ad-EGFP-Mieap Δ 275, and Ad-EGFP-Mieap Δ 496, respectively. FRAP experiments were performed on a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus), using a 60x/1.4 NA oil immersion objective (Olympus). Condensates were subjected to spot-bleaching or full-bleaching (bleaching entire condensates). For spot-bleaching, the bleaching area was unified to a diameter of 1.38 μ m. Condensates were imaged for 6 s, acquiring 30 images prior to spot-bleaching or 50 s, acquiring 5 images prior to full-bleaching. Photobleaching employed a 488-nm laser at 10% laser power with 11.6 μ s/ μ m exposure time or 1.4% laser power with 1.4 μ s/ μ m exposure time. Time-lapse images were acquired at 0.2 ms intervals for 60 s or 10 s intervals for 15 min. Spot-bleaching data for each construct were acquired from 15 different condensates. Full-bleaching data of each construct were acquired from 10 different condensates.

Expression and purification of GST and GST-Mieap

Escherichia coli (BL21) transformed with expression vectors were grown in 200 mL of Luria-Bertani medium at 37°C until the OD600 was between 0.55-0.6. Protein expression was induced with 100 μ M IPTG, and bacteria were subsequently incubated for 3 h at 25°C. After harvesting the bacteria by centrifugation at 3000 $\times g$ for 10 min at 4 °C, pellets were lysed with lysis buffer (1% Triton X-100 buffered in PBS supplemented with 1 mM Phenylmethylsulfonyl fluoride), and sonicated (20 $\times$ 30 s bursts with 10 s rest between bursts). Insoluble material was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. Supernatant was incubated with glutathione-Sepharose 4B (Cytiva) pre-equilibrated with

lysis buffer at 4°C overnight. After the beads were washed twice with lysis buffer, proteins were eluted with elution buffer (50 mM glutathione diluted in 50 mM Tris–HCl, pH 8.0), and dialyzed at 4°C overnight against PBS.

Lipid-binding analysis

For lipid-binding analysis, protein-lipid interactions on lipid-spotted membranes were evaluated with fat blot assays⁴⁴. Natural CL, PC, and PE derived from bovine heart (Olbracht Serdary Research Laboratories) were diluted with chloroform/methanol/1N HCl (80:80:1). 1 µL of each diluted lipid was spotted onto PVDF membranes (Cytiva) for antigen-antibody reactions using anti-Mieap antibody or nitrocellulose membranes (Cytiva) for antigen-antibody reactions using an anti-GST antibody to align spots with increasing amounts of lipids ranging from 0-667 pmol. Here, approximate molarities of CL, PC, and PE calculated from molecular weights of tetralinoleoyl CL, distearoyl PC, and distearoyl PC were used, respectively. After membranes were blocked with blocking buffer (3% fatty acid-free BSA diluted in 50 mM Tris–HCl, 150 mM NaCl, pH 7.5) for 1 h, membranes were incubated with 2.5 µg/mL of GST-Mieap or GST protein diluted in blocking buffer containing 0.1% Tween 20 overnight. Membranes were incubated with primary antibody (rabbit anti-Mieap antibody or rabbit anti-GST antibody) diluted in blocking buffer containing 0.06% Tween 20 (1:1000) for 3.5 h, and subsequently a secondary antibody (goat anti-rabbit antibody conjugated to horseradish-peroxidase) diluted in blocking buffer containing 0.06% Tween 20 (1:10000) for 1 h. ECL Western Blotting Detection Reagents (Cytiva) was used to detect HRP and chemiluminescence was visualized with an ImageQuant LAS 4000 system (Cytiva).

Lipid preparation

Lipid preparation was performed as described previously^{67,68}. Briefly, total lipids were extracted from samples using the Bligh-Dyer method⁶⁹. An aliquot of the organic phase was added to an equal volume of methanol before being loaded onto a DEAE-cellulose column (Wako Chemical) pre-equilibrated with chloroform. After successive washes with chloroform/methanol (1:1, v/v), acidic phospholipids were eluted with chloroform/methanol/HCl/water (12:12:1:1, v/v), followed by evaporation to dryness to yield a residue was soluble in methanol.

Mass spectrometric analyses of CL

Analyses were performed on a LC/MS/MS system consisting of a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source and an UltiMate 3000 system (Thermo Fisher Scientific). Lipid samples were separated on a Waters X-Bridge C₁₈ column (3.5 µm, 150 mm × 1.0 mm i.d.) at 40°C using a solvent step-gradient as follows: mobile phase A

(isopropanol/methanol/water (5:1:4, v/v/v) supplemented with 5 mM ammonium formate and 0.05% ammonium hydroxide (28% in water))/mobile phase B (isopropanol supplemented with 5 mM ammonium formate and 0.05% ammonium hydroxide (28% in water)) ratios of 60%/40% (0 min), 40%/60% (1 min), 20%/80% (9 min), 5%/95% (11-30 min), 95%/5% (31-35 min) and 60%/40% (45 min). Flow rate was 25 μ L/min. Source and ion transfer parameters applied were as follows. Spray voltage was 3.0 kV. For negative ionization modes, the sheath gas and capillary temperatures were maintained at 60 and 320 °C, respectively. The Orbitrap mass analyzer was operated at a resolving power of 70,000 in full-scan mode (scan range: 200–1800 m/z; automatic gain control (AGC) target:3e6) and of 35,000 in the Top 20 data-dependent MS2 mode (stepped normalized collision energy: 20, 30 and 40; isolation window: 4.0 m/z; AGC target: 1e5). Identification of CL molecular species was performed using LipidSearch 4.2 software (Mitsui Knowledge Industry)

Real-time ATP rate assay

LS174T-cont and Mieap-KD cells were seeded at a density of 2.5×10^4 cells/well (n=9) on a Seahorse XF24 Cell Culture Microplate. Cells were incubated at 37°C in a humidified chamber with 5% CO₂. 18 h after seeding, culture medium was replaced with XF DMEM medium pH 7.4 supplemented with 25 mM glucose and 2 mM L-glutamine through three washes. After cells were incubated at 37 °C in a non-CO₂ incubator for 60 min, cell culture plates were loaded into a Seahorse XFe24 Analyzer. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were recorded before and after serial injections of oligomycin and rotenone/antimycin A to yield final concentrations of 0.5 μ M.

Flow cytometric analysis

LS174T-cont and Mieap-KD cells cultured under normal conditions were harvested by trypsin-EDTA treatment. After adding complete growth media to inactivate trypsin, cells were centrifugated, washed with PBS, and incubated with 5 μ M 2',7'-dichlorofluorescein-diacetate (Sigma) for 20 min at 37°C. After being washed with PBS, cells were immediately analyzed with an EC800 flow cytometry analyzer (Sony) using the 488-nm line.

Quantification and statistical analysis

FRAP data

Fluorescence recovery rates were calculated using cellSens Imaging Software (Olympus), in which the intensity initially acquired after bleaching was set to 0 and the pre-bleaching intensity was set to 1. The normalized average fluorescence recovery was plotted in JMP 14.2.0 (SAS).

Crista data

For quantification of crista data, crista area and outlines of mitochondrial sections in TEM images were marked manually using Adobe Photoshop CC, where normal crista morphology was identified by the presence of lamellar structures with distinct osmium staining. Aberrant crista-like structures that were not observed in mitochondria of WT were excluded. Subsequently, the ratio of crista area per mitochondrial section was calculated from the indicated number of mitochondria in legends of Fig. 6D, 6I, 6K and 7F, using Image J⁷⁰, and violin plots were generated in JMP 14.2.0 (SAS). Statistical analysis was conducted using Student's two-tailed t-tests.

Declarations

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Author contributions

NI, KH, YS, YN, and HA performed experiments. NI and HA analyzed data. NI and HA designed the research. NI and HA wrote paper. HA coordinated and supervised the project.

Declaration of interests

The authors declare no competing interests.

References

1. Shin, Y., and Brangwynne, C.P. Liquid phase condensation in cell physiology and disease. *Science* **357**, eaaf4382 (2017).
2. Boeynaems, S., Alberti, S., Fawzi, N.L., Mittag, T., Polymenidou, M., Rousseau, F., Schymkowitz, J., Shorter, J., Wolozin, B., Van Den Bosch, L., et al. Protein Phase Separation: A New Phase in Cell Biology.

Trends Cell Biol. **28**, 420–435 (2018).

3. Alberti, S., Gladfelter, A., and Mittag, T. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. *Cell* **176**, 419–434 (2019).

4. Sabari, B.R., Dall'Agnese, A., Boija, A., Klein, I.A., Coffey, E.L., Shrinivas, K., Abraham, B.J., Hannett, N.M., Zamudio, A.V., Manteiga, J.C., et al. Coactivator condensation at super-enhancers links phase separation and gene control. *Science* **361**, eaar3958 (2018).

5. Case, L.B., Zhang, X., Ditlev, J.A., and Rosen, M.K. Stoichiometry controls activity of phase-separated clusters of actin signaling proteins. *Science* **363**, 1093–1097 (2019).

6. Huang, W.Y.C., Alvarez, S., Kondo, Y., Lee, Y.K., Chung, J.K., Lam, H.Y.M., Biswas, K.H., Kuriyan, J., and Groves, J.T. A molecular assembly phase transition and kinetic proofreading modulate Ras activation by SOS. *Science* **363**, 1098–1103 (2019).

7. Du, M.J., and Chen, Z.J.J. DNA-induced liquid phase condensation of cGAS activates innate immune signaling. *Science* **361**, 704–709 (2018).

8. Woodruff, J.B., Gomes, B.F., Widlund, P.O., Mahamid, J., Honigsmann, A., and Hyman, A.A. The centrosome is a selective condensate that nucleates microtubules by concentrating tubulin. *Cell* **169**, 1066–1077 (2017).

9. Rai, A.K., Chen, J.X., Selbach, M., and Pelkmans, L. Kinase-controlled phase transition of membraneless organelles in mitosis. *Nature* **559**, 211–216 (2018).

10. Welch, G.R. On the role of organized multienzyme systems in cellular metabolism: a general synthesis. *In Prog. Biophys. Mol. Biol.* (London, UK: Elsevier), pp. 103–191 (1977).

11. Srere, P.A. COMPLEXES OF SEQUENTIAL METABOLIC ENZYMES. *Annu. Rev. Biochem.* **56**, 89–124 (1987).
12. Jin, M.Y., Fuller, G.G., Han, T., Yao, Y., Alessi, A.F., Freeberg, M.A., Roach, N.P., Moresco, J.J., Karnovsky, A., Baba, M., et al. Glycolytic Enzymes Coalesce in G Bodies under Hypoxic Stress. *Cell Rep.* **20**, 895–908 (2017).
13. Lee, H., DeLoache, W.C., and Dueber, J.E. Spatial organization of enzymes for metabolic engineering. *Metab. Eng.* **14**, 242–251 (2012).
14. Kohnhorst, C.L., Kyoung, M., Jeon, M., Schmitt, D.L., Kennedy, E.L., Ramirez, J., Bracey, S.M., Luu, B.T., Russell, S.J., and An, S. Identification of a multienzyme complex for glucose metabolism in living cells. *J. Biol. Chem.* **292**, 9191–9203 (2017).
15. Schmitt, D.L., and An, S. Spatial Organization of Metabolic Enzyme Complexes in Cells. *Biochemistry* **56**, 3184–3196 (2017).
16. An, S.G., Kumar, R., Sheets, E.D., and Benkovic, S.J. Reversible compartmentalization of de novo purine biosynthetic complexes in living cells. *Science* **320**, 103–106 (2008).
17. Macfarlane, M.G. Structure of cardiolipin. *Nature* **182**, 946 (1958).
18. Ikon, N., and Ryan, R.O. Cardiolipin and mitochondrial cristae organization. *Biochim. Biophys. Acta (BBA)-Biomembranes* **1859**, 1156–1163 (2017).
19. Paradies, G., Paradies, V., De Benedictis, V., Ruggiero, F.M., and Petrosillo, G. Functional role of cardiolipin in mitochondrial bioenergetics. *Biochim. Biophys. Acta (BBA)-Bioenergetics* **1837**, 408–417 (2014).

20. Ren, M.D., Phoon, C.K.L., and Schlame, M. Metabolism and function of mitochondrial cardiolipin. *Prog. Lipid Res.* **55**, 1–16 (2014).
21. Potting, C., Tatsuta, T., König, T., Haag, M., Wai, T., Aaltonen, M.J., and Langer, T. TRIAP1/PRELI complexes prevent apoptosis by mediating intramitochondrial transport of phosphatidic acid. *Cell Metab.* **18**, 287–295 (2013).
22. Ye, C., Shen, Z., and Greenberg, M.L. Cardiolipin remodeling: a regulatory hub for modulating cardiolipin metabolism and function. *J. Bioenerg. Biomembr.* **48**, 113–123 (2016).
23. Paradies, G., Paradies, V., Ruggiero, F.M., and Petrosillo, G. Role of cardiolipin in mitochondrial function and dynamics in health and disease: Molecular and pharmacological aspects. *Cells* **8**, 728 (2019).
24. Saric, A., Andreau, K., Armand, A.S., Moller, I.M., and Petit, P.X. Barth syndrome: from mitochondrial dysfunctions associated with aberrant production of reactive oxygen species to pluripotent stem cell studies. *Front Genet.* **6**: 359 (2016)
25. Acehan, D., Xu, Y., Stokes, D.L. and Schlame, M. Comparison of lymphoblast mitochondria from normal subjects and patients with Barth syndrome using electron microscopic tomography. *Lab Invest.* **87**, 40-48 (2006).
26. Dudek, J., Cheng, I.F., Balleininger, M., Vaz, F.M., Streckfuss-Bomeke, K., Hubscher, D., Vukotic, M., Wanders, R.J., Rehling, P. and Guan, K. Cardiolipin deficiency affects respiratory chain function and organization in an induced pluripotent stem cell model of Barth syndrome. *Stem Cell Res.* **11**, 806-819 (2013).
27. Miyamoto, Y., Kitamura, N., Nakamura, Y., Futamura, M., Miyamoto, T., Yoshida, M., Ono, M., Ichinose, S., and Arakawa, H. Possible Existence of Lysosome-Like Organella within Mitochondria and Its Role in Mitochondrial Quality Control. *PLoS One* **6**, e16054 (2011).

28. Kamino, H., Nakamura, Y., Tsuneki, M., Sano, H., Miyamoto, Y., Kitamura, N., Futamura, M., Kanai, Y., Taniguchi, H., Shida, D., et al. Mieap-regulated mitochondrial quality control is frequently inactivated in human colorectal cancer. *Oncogenesis* **5**, e181 (2016).
29. Okuyama, K., Kitajima, Y., Egawa, N., Kitagawa, H., Ito, K., Aishima, S., Yanagihara, K., Tanaka, T., and Noshiro, H. Mieap-induced accumulation of lysosomes within mitochondria (MALM) regulates gastric cancer cell invasion under hypoxia by suppressing reactive oxygen species accumulation. *Sci. Rep.* **9**, 2822 (2019).
30. Tsuneki, M., Nakamura, Y., Kinjo, T., Nakanishi, R., and Arakawa, H. Mieap suppresses murine intestinal tumor via its mitochondrial quality control. *Sci. Rep.* **5**, 12472 (2015).
31. Mussazhanova, Z., Shimamura, M., Kurashige, T., Ito, M., Nakashima, M., and Nagayama, Y. Causative role for defective expression of mitochondria-eating protein in accumulation of mitochondria in thyroid oncocytic cell tumors. *Cancer Sci.* **111**, 2814–2823 (2020).
32. Gaowa, S., Futamura, M., Tsuneki, M., Kamino, H., Tajima, J.Y., Mori, R., Arakawa, H., and Yoshida, K. Possible role of p53/Mieap-regulated mitochondrial quality control as a tumor suppressor in human breast cancer. *Cancer Sci.* **109**, 3910–3920 (2018).
33. Kitamura, N., Nakamura, Y., Miyamoto, Y., Miyamoto, T., Kabu, K., Yoshida, M., Futamura, M., Ichinose, S., and Arakawa, H. Mieap, a p53-Inducible Protein, Controls Mitochondrial Quality by Repairing or Eliminating Unhealthy Mitochondria. *PLoS One* **6**, e16060 (2011).
34. Kriventseva, E.V., Kuznetsov, D., Tegenfeldt, F., Manni, M., Dias, R., Simao, F.A., and Zdobnov, E.M. OrthoDB v10: sampling the diversity of animal, plant, fungal, protist, bacterial and viral genomes for evolutionary and functional annotations of orthologs. *Nucleic Acids Res.* **47**, D807–D811 (2019).

35. Huang, Y.J., Acton, T.B., and Montelione, G.T. DisMeta: a meta server for construct design and optimization. *Methods Mol. Biol.* **1091**, 3–16 (2014).
36. Uversky, V.N., Radivojac, P., Iakoucheva, L.M., Obradovic, Z., and Dunker, A.K. Prediction of intrinsic disorder and its use in functional proteomics. *Methods Mol. Biol.* **408**, 69–92 (2007).
37. Lupas, A., Van Dyke, M., and Stock, J. Predicting coiled coils from protein sequences. *Science* **252**, 1162–1164 (1991).
38. Holehouse, A.S., Das, R.K., Ahad, J.N., Richardson, M.O., and Pappu, R.V. CIDER: Resources to Analyze Sequence-Ensemble Relationships of Intrinsically Disordered Proteins. *Biophys. J.* **112**, 16–21 (2017).
39. Kyte, J., and Doolittle, R.F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132 (1982).
40. Kaewsuya, P., Danielson, N.D., and Ekhterae, D. Fluorescent determination of cardiolipin using 10-N-nonyl acridine orange. *Anal. Bioanal. Chem.* **387**, 2775–2782 (2007).
41. Sathappa, M. and Alder, N.N. The ionization properties of cardiolipin and its variants in model bilayers. *Biochim. Biophys. Acta.* **1858**, 1362-1372 (2016).
42. Belazi, D., Sole-Domenech, S., Johansson, B., Schalling, M., and Sjoval, P. Chemical analysis of osmium tetroxide staining in adipose tissue using imaging ToF-SIMS. *Histochem. Cell Biol.* **132**, 105–115 (2009).
43. Planas-Iglesias, J., Dwarakanath, H., Mohammadyani, D., Yanamala, N., Kagan, V.E., and Klein-Seetharaman, J. Cardiolipin Interactions with Proteins. *Biophys. J.* **109**, 1282–1294 (2015).

44. Miyamoto, T., Kitamura, N., Ono, M., Nakamura, Y., Yoshida, M., Kamino, H., Murai, R., Yamada, T., and Arakawa, H. Identification of 14-3-3 γ as a MIEAP-interacting protein and its role in mitochondrial quality control. *Sci. Rep.* **2**, 379 (2012).
45. Munnik, T., and Wierchowicka, M. Lipid-binding analysis using a fat blot assay. *Methods Mol. Biol.* **1009**, 253–259 (2013).
46. Bota, D.A., and Davies, K.J. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat. Cell Biol.* **4**, 674–680 (2002).
47. Choi, S.Y., Huang, P., Jenkins, G.M., Chan, D.C., Schiller, J., and Frohman, M.A. A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nat. Cell Biol.* **8**, 1255–1262 (2006).
48. Lee, J.H., Park, A., Oh, K.-J., Lee, S.C., Kim, W.K., and Bae, K.-H. The role of adipose tissue mitochondria: Regulation of mitochondrial function for the treatment of metabolic diseases. *Int. J. Mol. Sci.* **20**, 4924 (2019).
49. Sidossis, L. and Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest.* **125**, 478–486 (2015).
50. Fenzl, A. and Kiefer, F.W. Brown adipose tissue and thermogenesis. *Horm Mol Biol Clin Invest.* **19**, 25–37 (2014).
51. Crichton, P.G., Lee, Y., and Kunji, E.R. The molecular features of uncoupling protein 1 support a conventional mitochondrial carrier-like mechanism. *Biochimie.* **134**, 35–50 (2017).
52. Castellana, M., Wilson, M.Z., Xu, Y.F., Joshi, P., Cristea, I.M., Rabinowitz, J.D., Gitai, Z., and Wingreen, N.S. Enzyme clustering accelerates processing of intermediates through metabolic channeling. *Nat.*

Biotechnol. **32**, 1011–1018 (2014).

53. Prouteau, M., and Loewith, R. Regulation of Cellular Metabolism through Phase Separation of Enzymes. *Biomolecules* **8**, 160 (2018).

54. Strulson, C.A., Molden, R.C., Keating, C.D., and Bevilacqua, P.C. RNA catalysis through compartmentalization. *Nat. Chem.* **4**, 941–946 (2012).

55. Bracha, D., Walls, M.T., and Brangwynne, C.P. Probing and engineering liquid-phase organelles. *Nat. Biotechnol.* **37**, 1435–1445 (2019).

56. Zhao, E.M., Suek, N., Wilson, M.Z., Dine, E., Pannucci, N.L., Gitai, Z., Avalos, J.L., and Toettcher, J.E. Light-based control of metabolic flux through assembly of synthetic organelles. *Nat. Chem. Biol.* **15**, 589–597 (2019).

57. Hinzpeter, F., Gerland, U., and Tostevin, F. Optimal Compartmentalization Strategies for Metabolic Microcompartments. *Biophys. J.* **112**, 767–779 (2017).

58. Kojima, T., and Takayama, S. Membraneless Compartmentalization Facilitates Enzymatic Cascade Reactions and Reduces Substrate Inhibition. *ACS Appl. Mater. Interfaces* **10**, 32782–32791 (2018).

59. Dan, X., Babbar, M., Moore, A., Wechter, N., Tian, J., Mohanty, J.G., Croteau, D.L., and Bohr, V.A. DNA damage invokes mitophagy through a pathway involving Spata18. *Nucleic Acids Res.* **48**, 6611–6623 (2020).

60. Tatsuta, T. and Langer, T. Quality control of mitochondria: protection against neurodegeneration and aging. *EMBO J.* **27**, 306–314 (2008).

61. Oshima, H., Matsunaga, A., Fujimura, T., Tsukamoto, T., Taketo, M.M., and Oshima, M. Carcinogenesis in mouse stomach by simultaneous activation of the Wnt signaling and prostaglandin E-2 pathway. *Gastroenterology* **131**, 1086–1095 (2006).
62. Serricchio, M., Vissa, A., Kim, P.K., Yip, C.M., and McQuibban, G.A. Cardiolipin synthesizing enzymes form a complex that interacts with cardiolipin-dependent membrane organizing proteins. *Biochim. Biophys. Acta-Mol. Cell Biol. Lipids* **1863**, 447–457 (2018).
63. Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., and Green, D.R. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* **2**, 156–162 (2000).
64. Nakamura, Y., Kitamura, N., Shinogi, D., Yoshida, M., Goda, O., Murai, R., Kamino, H., and Arakawa, H. BNIP3 and NIX mediate Mieap-induced accumulation of lysosomal proteins within mitochondria. *PLoS One* **7**, e30767 (2012).
65. Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., *et al.* p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* **102**, 849–862 (2000).
66. Wilkins, M.R., Gasteiger, E., Bairoch, A., Sanchez, J.C., Williams, K.L., Appel, R.D., and Hochstrasser, D.F. Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol.* **112**, 531–552 (1999).
67. Shindou, H., Koso, H., Sasaki, J., Nakanishi, H., Sagara, H., Nakagawa, K.M., Takahashi, Y., Hishikawa, D., Iizuka-Hishikawa, Y., Tokumasu, F., *et al.* Docosahexaenoic acid preserves visual function by maintaining correct disc morphology in retinal photoreceptor cells. *J. Biol. Chem.* **292**, 12054–12064 (2017).
68. Yamamoto, T., Endo, J., Kataoka, M., Matsushashi, T., Katsumata, Y., Shirakawa, K., Yoshida, N., Isobe, S., Moriyama, H., Goto, S., *et al.* Decrease in membrane phospholipids unsaturation correlates with myocardial diastolic dysfunction. *PLoS One* **13**, e0208396 (2018).

69. Bligh, E.G., and Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem.* **37**, 911–917 (1959).

70. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).

Supporting Information

●Supplemental tables

- Table S1. Mass spectrometric data of CL under exogenous and physiological expression of Mieap
- Table S2. Oligonucleotides used in this paper

●Supplemental figures

- Figure S1. Mieap forms liquid droplets
- Figure S2. Analyses of Mieap orthologs
- Figure S3. FRAP analysis of condensates formed by EGFP-Mieap and three deletion mutants
- Figure S4. Verification of subcellular localization of fluorescently-labeled proteins
- Figure S5. Verification of subcellular localization of fluorescently-labeled proteins
- Figure S6. Hypothetical models for biosurfactant activity of Mieap and Mieap-mediated sequential enzymatic reactions in CL metabolism

●Supplemental Movies

- Video S1. Time-lapse imaging of A549-cont cells expressing EGFP-Mieap, co-stained with MitoTracker Red
- Video S2. Time-lapse imaging of self-fusing condensates comprised of EGFP-Mieap in an A549-cont cell
- Video S3. Z-stack, 3D, and time-lapse imaging, showing the spatial relationship between MLDs and mApple-TOMM20 in A549-cont cells
- Video S4. Z-stack imaging of condensates comprised of EGFP-Mieap, EGFP-Mieap Δ CC, EGFP-Mieap Δ 275, or EGFP-Mieap Δ 496 in A549-cont cells, co-stained with MitoTracker Red or unstained

- Video S5. Z-stack and/or time-lapse imaging for Mi-LDs and fluorescence probes, including EGFP-BNIP3, EGFP-NIX, AcGFP1-Mito, DsRed2-Mito, and SYBR Green I in A549 cells
- Video S6. Z-stack and/or time-lapse imaging for Mi-LDs and fluorescence probes including 10-nonylacridine orange bromide (NAO), EGFP-cytochrome *c*, EGFP-ATP5F1B, and EGFP-PHB2, in A549-cont cells
- Video S7. Z-stack imaging for Mi-LDs and fluorescence probes, including EGFP- TAMM41, EGFP-PGS1, EGFP-PTPMT1, EGFP-CRLS1, EGFP-PLA2G6, EGFP-TAZ, EGFP-PRELI, EGFP-LONP1, and EGFP-PLD6, in A549-cont cells

Figures

Figure 1

Figure 1

Mieap forms liquid droplets in mitochondria. (A) Confocal microscopy showing the spatial relationship between Mi-LDs (EGFP-Mieap) and mitochondrial outer membranes visualized with mApple-TOMM20. Left: a cell image. Middle: higher magnification of the area indicated by the dashed square on the left panel and a line-scan of fluorescence intensities along the dashed arrow. Right: 3D reconstruction of the cell shown in the left panel. See also Video S3. (B) Z-stack images of the cell in (A). (C) Super-resolution

images showing the spatial relationship between Mi-LDs (EGFP-Mieap) and mitochondrial outer membranes, visualized with mApple-TOMM20. (D) Confocal microscopy showing the spatial relationship between enlarged Mi-LDs and mApple-TOMM20. Lower right: line-scan as in (A). See also Video S3.

Figure 2

Figure 2

Mieap is an IDR-containing protein (A) Phylogenetic spread of Mieap orthologs annotated with OrthoDB v10. Red sectors indicate present species. Light blue sectors indicate missing species. (B) Proportion of

amino acid residues in each domain of the Mieap protein. (C) Multiple sequence alignment for Mieap orthologs in representative eukaryotes. Black and gray boxes indicate 100% and 80% identical residues among eukaryotes, respectively. Blue letters indicate IDRs annotated by VL3-BA. Orange letters indicate coiled-coil regions annotated by COILS. (D) Schematic of the domain structure of Mieap. The dashed vertical line indicates the boundary of gross hydrophilic and hydrophobic halves, separated by IDR3 and the adjacent structured region. Asterisks indicate clusters of positively charged residues. (E) Sequence analyses of Mieap protein. VL3-BA prediction of IDRs on the amino acid sequence of Mieap, in which bold lines indicate IDRs; DisMeta, meta-prediction of IDRs on the amino acid sequence of Mieap; COILS; coiled-coil regions annotated on the amino acid sequence of Mieap using a 21-residue sliding window; Hydro, hydrophobicity of Mieap using a 9-residue sliding window; NCPR, the linear net charge per residue of Mieap using a 5-residue sliding window.

Figure 3

Figure 3

Material state and dynamics of Mi-LDs are determined by specific regions of Mieap. (A) EGFP-Mieap and the three deletion-mutant forms. The schematic indicates wild-type (WT) and three deletion mutants (Δ CC, Δ 275, and Δ 496) of EGFP-Mieap protein. Numbers indicate amino acid residues. (B) Representative images with a cross-sectional slice view of LDs formed by WT, Δ CC, Δ 275, and Δ 496 in A549-cont cells obtained with fluorescent confocal microscopy. See also Video S4. (C) Subcellular localization of LDs

formed by WT, Δ CC, Δ 275, and Δ 496, compared with a mitochondrial probe MitoTracker Red. See also Video S4. (D) Normalized average fluorescence recovery in the FRAP experiment. Condensates formed by WT, Δ CC, Δ 275, and Δ 496 were analyzed in A549-cont cells. Each condensate was subjected to spot-bleaching using a 488-nm laser at 10% laser power with 11.6 μ s/ μ m exposure time and followed up for 60 s. n = 15 condensates for each construct. Data shown are means $\pm$ SD. (E) Normalized average fluorescence recovery in the FRAP experiment with weaker laser exposure as in (D). Laser power was weakened to 1.4% and exposure time was shortened to 1.4 μ s/ μ m. Observation duration was expanded to 15 min after photobleaching entire condensates. n = 10 condensates for each construct. Data shown are means $\pm$ SD.

Figure 4

Figure 4

Mi-LDs phase-separate mitochondrial CL and CL-binding proteins. (A-I) Live-cell imaging of Mi-LDs and various mitochondrial fluorescence probes in A549 cells. Whether each mitochondrial fluorescence probe is phase-separated by Mi-LDs was examined with live-cell imaging analysis in A549 cells. EGFP-BNIP3 (A), EGFP-NIX (B), AcGFP-mito (C), DsRed2-mito (D), and SYBR Green I (E), were not incorporated into Mi-LDs. However, 10-nonylacridine orange bromide (NAO) (F), EGFP-cytochrome c (G), EGFP-ATP5F1B (H),

and EGFP-PHB2 (I) were incorporated into Mi-LDs. Lower right: line-scan of fluorescence intensities along the dashed arrow. See also Videos S5 and S6. (J) Lipid-binding analysis of GST-tagged Mieap protein. GST-Mieap or GST was incubated with membranes on which increasing amounts of CL, phosphatidylcholine (PC), and phosphatidylethanolamine (PE), ranging from 0-667 pmol, were spotted. Protein-lipid interactions were visualized using an anti-Mieap antibody and/or anti-GST antibody, as indicated.

Figure 5

Figure 5

Mi-LDs compartmentalize and facilitate CL metabolic reactions. (A) Quantitative assessment of total CL by mass spectrometric analysis. Uninfected A549 cells or cells infected with Ad-Mieap were subjected to mass spectrometric analysis for CL. Duplicate experiments were performed. Data shown are means $\pm$ SD. (B) Quantitative and rate assessments of CL species by mass spectrometric analysis. A549 cells were analyzed as described in (A). Absolute values of selected CL species were shown as amounts of substance per cell (left panel). Relative values of selected CL species were shown as % of total CL (right panel). Data shown are means $\pm$ SD. (C) The conventional CL metabolic pathway. Abbreviations: PA, phosphatidic acid; CDP-DG: cytidine diphosphate diacylglycerol; PGP, phosphatidylglycerophosphate; PG, phosphatidylglycerol; CLN, nascent cardiolipin; CLM, mature cardiolipin. (D-J) Live-cell imaging of Mi-LDs and CL metabolic enzymes. EGFP-TAMM41 (D), EGFP-PGS1 (E), EGFP-PTPMT1 (F), EGFP-CRLS1 (G), EGFP-PLA2G6 (H), EGFP-TAZ (I), and EGFP-PRELI (J) were incorporated into Mi-LDs. Lower right: line-scan of fluorescence intensities along the dashed arrow. See also Video S7. (K, L) Live-cell imaging of Mi-LDs and LONP1/PLD6. EGFP-LONP1 (K) and EGFP-PLD6 (L) were not incorporated into Mi-LDs. Lower right: line-scan of fluorescence intensities along the dashed arrow. See also Video S7.

Figure 6

Figure 6

Mieap may promote mitochondrial quality control by regulating CL metabolism. (A) Oxygen consumption rates (OCR) of LS174T-cont and Mieap-KD cells under normal conditions calculated with a flux analyzer. Data are shown as means $\pm$ SD (n = 9). Significance was assessed using Student's two-tailed t-test (p < 0.01). (B) Mitochondrial ATP production rates of LS174T-cont and Mieap-KD cells under normal conditions calculated with a flux analyzer, using a Seahorse XF real-time ATP rate assay. Data are shown

as means $\pm$ SD (n = 9). Significance was assessed using Student's two-tailed t-test ($p < 0.01$). (C) Morphology of mitochondria of LS174T-cont and Mieap-KD cells with transmission electron microscopy (TEM). (D) Ratio of crista area per mitochondrial section of LS174T-cont and Mieap-KD cells. Quantitative data were obtained from cont mitochondria (n=197) and Mieap-KD mitochondria (n= 329) in TEM images and displayed in violin plot. Significance was assessed using Student's two-tailed t-test ($p < 0.0001$). (E) Reactive oxygen species (ROS) levels of LS174T-cont and Mieap-KD cells cultured under normal conditions, analyzed by flow cytometry using 2',7'-dichlorofluorescein-diacetate. Data from duplicate experiments are shown. (F) Quantitative assessment of total CL by mass spectrometric analysis. LS174T cells with (Cont) and without (Mieap-KD) endogenous Mieap expression were subjected to mass spectrometric analysis. Duplicate experiments were performed. Data shown are means $\pm$ SD. (G) Quantitative and rate assessments of CL species by mass spectrometric analysis. LS174T cells were analyzed as described in (F). Absolute values of selected CL species are shown as the amount of substance per cell (left panel). Relative values of selected CL species are shown as % of total CL (right panel). Data shown are means $\pm$ SD. See also Table S1. (H) Morphology of kidney mitochondria of Mieap+/+ and Mieap-/- mice with TEM. (I) Ratios of crista area per mitochondrial section of Mieap+/+ and Mieap-/- mouse kidneys. Quantitative data were obtained from Mieap+/+ kidney mitochondria (n=190) and Mieap-/- kidney mitochondria (n= 234) in TEM images and displayed in a violin plot. Significance was assessed using Student's two-tailed t-test ($p < 0.0001$). (J) Morphology of liver mitochondria of Mieap+/+ and Mieap-/- mice with TEM. (K) Ratios of crista area per mitochondrial section of Mieap+/+ and Mieap-/- mouse livers. Quantitative data were obtained from Mieap+/+ liver mitochondria (n=146) and Mieap-/- liver mitochondria (n= 134) in TEM images and displayed in a violin plot. Significance was assessed using Student's two-tailed t-test ($p < 0.0001$). (L, M) Comparison of absolute values of CL species between Mieap+/+ and Mieap-/- mouse kidney (L) and liver (M). Data shown are means $\pm$ SD. See also Table S1.

Figure 7

Figure 7

Mieap prevents obesity by regulating CL metabolism. (A) Body weights of 1,353 Mieap^{+/+}, Mieap^{+/-}, and Mieap^{-/-} mice (n = 364 Mieap^{+/+}, 656 Mieap^{+/-}, and 333 Mieap^{-/-} mice, 7-130 weeks of age) were weighed. Dots and quadratic regression curves with 95% confidence intervals are shown for each genotype. (B) Body weights of Mieap^{+/+}, Mieap^{+/-}, and Mieap^{-/-} mice (7-130 weeks of age) in (A). Data shown are means ±SE. Significance was assessed using Student's two-tailed t-test (*p < 0.05). (C) Body

weights of *Mieap*^{+/+}, *Mieap*^{+/-}, and *Mieap*^{-/-} mice from middle-age and older mice (44-104 weeks of age) in (A). Data shown are means $\pm$ SE. Significance was assessed using Student's two-tailed t-test (* $p < 0.05$, *** $p < 0.001$). (D) Body weights of *Mieap*^{+/+}, *Mieap*^{+/-}, and *Mieap*^{-/-} mice around 60 (53-62) weeks of age in (A). Data shown are means $\pm$ SE. Significance was assessed using Student's two-tailed t-test (* $p < 0.05$, *** $p < 0.001$). (E) Morphology of brown adipose tissue (BAT) mitochondria of *Mieap*^{+/+} and *Mieap*^{-/-} mice with TEM. (F) The ratio of crista area per mitochondrial section of *Mieap*^{+/+} and *Mieap*^{-/-} mice BAT. Quantitative data were obtained from *Mieap*^{+/+} BAT mitochondria (n=181) and *Mieap*^{-/-} BAT mitochondria (n=129) in TEM images and displayed in a violin plot. Significance was assessed using Student's two-tailed t-test ($p < 0.0001$). (G) A hypothetical model for mitochondrial quality control via the *Mieap*-MLOs-CL metabolism axis.

Supplementary Files

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